ON THE METABOLISM OF SULPHUR IN EXCISED ROOTS.

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by

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Summary

1. The assimilation of several sulphur compounds in excised roots grown in culture was studied. To obtain sulphate deficient conditions a sulphate impurity was removed from the sugar component of the medium by ion exchange. The sulphur compounds investigated were sulphate, taurine, cysteic acid, elemental sulphur, cystine, cystamine, glutathione, homocystine and methionine. Of these possible sources of sulphur, sulphate, cystine, homocystine and methionine were utilized for growth. All four were available for the synthesis of protein cysteine and methionine. The roots responded to elemental sulphur but whether the roots assimilated the sulphur before or after oxidation to sulphate was not established.

2. The radioactive sulphur compounds present in tomato root extracts were separated after ion exchange fractionation by thin layer electrophoresis and chromatography. Thiols present in these extracts were protected from oxidation by reaction with iodoacetamide, but acetamidocysteine was later found to be unstable.

3. Among the compounds labelled by incubating tomato roots for quarter of an hour with carrier-free (^{35}S)sulphate, were glutathione, cysteine and methionine as well as protein cysteine and methionine.

4. When both sulphate and methionine were supplied to roots, exogenous methionine was incorporated preferentially into protein methionine whereas the label of sulphate, after assimilation into cysteine was incorporated preferentially into protein cysteine. Glutathione was but slightly labelled and cysteine not at all by methionine in the presence of sulphate so sulphate or a metabolite reduced the conversion of methionine into cysteine and supplied most of the sulphur for cysteine synthesis.

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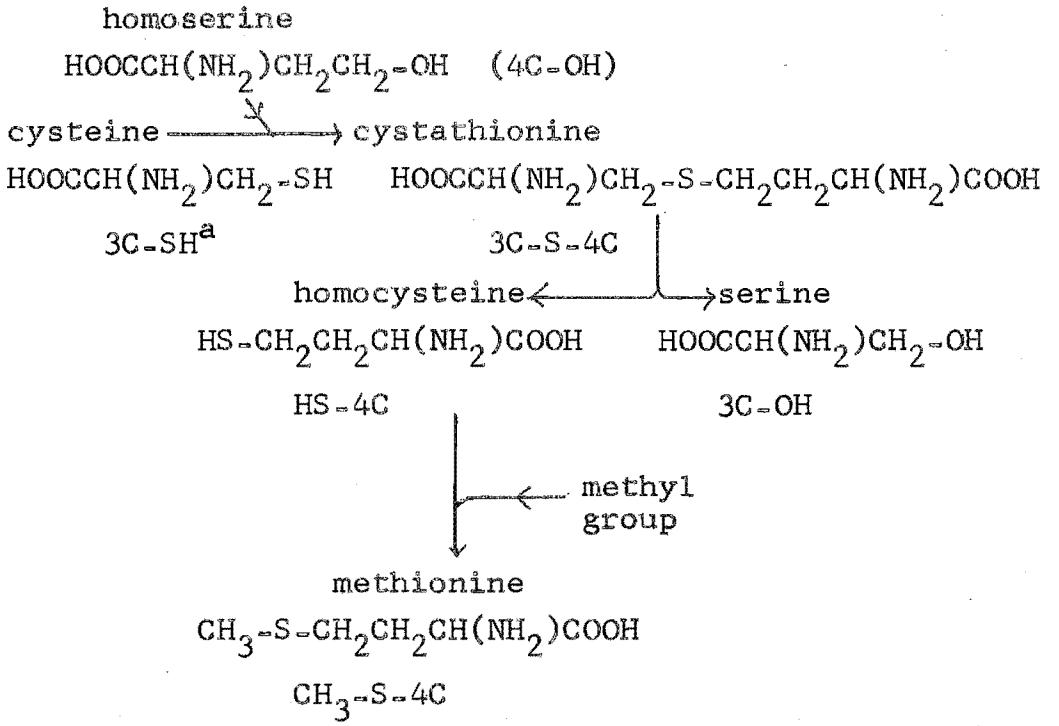
Chapter 1

Introduction

Sulphur is an essential element in the nutrition of higher plants and is usually assimilated as sulphate ion. As well as sulphate, several organic sulphur containing compounds have been reported in plants and of these the amino acids cysteine, cystine and methionine are essential structural elements of many proteins.

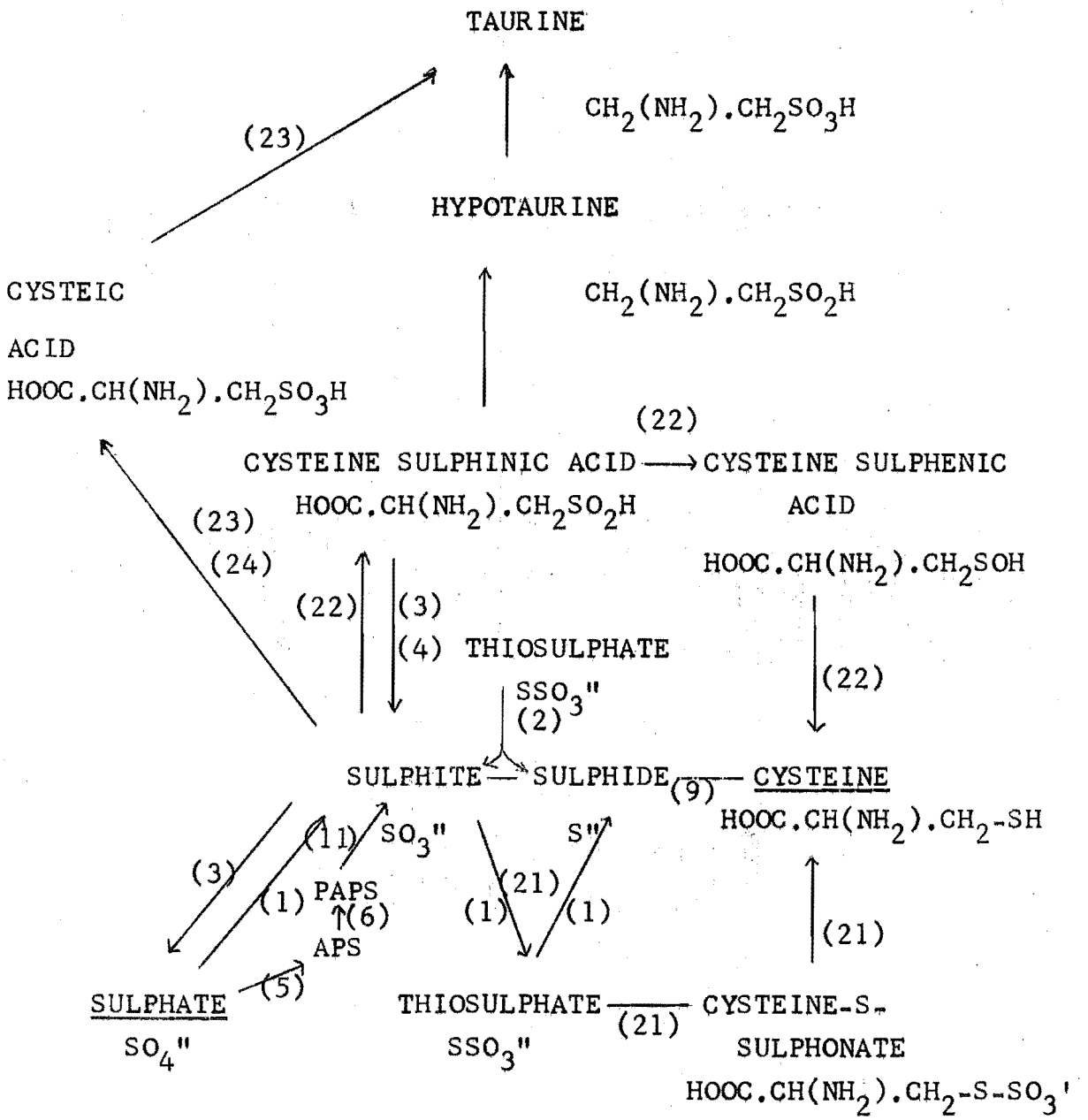
Other sulphur containing compounds such as γ-glutamyl peptides, the thioglucosides and isothiocyanates, and the sulphur compounds found in the alliums, can be present in large quantities but they are of restricted distribution and for many plants the protein amino acids are quantitatively the most important.

The formation of cysteine and methionine involves the reduction of sulphate and the incorporation of an inorganic compound into an organic one. In higher plants, Wilson (1962) maintained that, with few exceptions the location and mechanism of sulphate reduction was unknown, while in 1964 Davies, Giovanelli and Rees concluded that for these plants knowledge of the pathway of sulphate reduction was fragmentary. Davies et al. summarized some recent work to suggest that after reduction from sulphate (+6) to sulphide (-2) through sulphite (+4), inorganic sulphur first entered into organic combination in cysteine, a three carbon α-amino acid, but they also suggested that entry into homocysteine, a four carbon α-amino acid, was equally likely. Since the metabolic relationships between cysteine and methionine were unknown, they suggested, from studies on fungi, that after incorporation into cysteine, the sulphur atom would be transfered to homocysteine through cystathionine. From homocysteine, a thiol or -SH compound, is derived methionine by methylation of the thiol group to give a thioether.



(a) The convention - 3C-SH, 4C-SH etc. was adopted since a 3C and a 4C chain differ only in an extra (CH_2) group, being α-amino acids. All the compounds are of the L-configuration.

Since the assimilation of sulphate into cysteine and methionine is quantitatively most important and an interest in sulphur as a plant nutrient has recently been stimulated by the establishment of widespread sulphur deficiencies, an investigation of sulphate assimilation by higher plants was undertaken.



Several of the possible reactions involved in the synthesis and metabolism of cysteine.

The numbers refer to equations in the text where the reactions are discussed.

The Pathways of sulphate assimilation

The assimilation of sulphate by bacteria

Although the formation of sulphite (SO_3") from sulphate (SO_4") is now the most well known section of the sulphate reduction pathway, the position of sulphite as an intermediate in the reduction of sulphate has been established much earlier in Escherichia coli by Lampen, Roepke and Jones (1947). They isolated a series of mutants unable to assimilate sulphate; these they arranged in the following order from nutritional studies: sulphiteless (that is requiring sulphite for growth and unable to grow on sulphate), thiosulphateless - sulphideless (these last two types utilized either thiosulphate or sulphide), cysteineless, cystathionineless, homocysteineless and methionineless. Each type is able to utilize the compounds available to the mutants following it. Cowie, Bolton and Sands (1950) and also Roberts, Abelson, Cowie, Bolton and Britten (1955) studied the inhibition of (^{35}S) sulphate uptake brought about by the simultaneous addition of either sulphite, thiosulphate, cysteic acid, taurine, sulphide, cysteine, homocysteine and methionine to growing cultures of E. coli. Taurine and cysteic acid had no effect on the assimilation of sulphate. Cysteine, sulphite and methionine could inhibit uptake almost entirely and homocysteine could inhibit partially at the concentrations tried. From these studies they concluded that the pathway of sulphate reduction was predominantly inorganic in that assimilation into an organic compound did not take place until the -2 level of oxidation in sulphide (and also in cysteine and methionine) had been reached. On the basis of oxidation levels they arranged the compounds in the following order during sulphate reduction:

sulphate	→ sulphite	→ thiosulphate	→ sulphide	→ cysteine
SO_4"	SO_3"	SSO_3"	S"	$HOOCCH(NH_2)CH_2SH$

(1)

Thus thiosulphate was considered to be an intermediate.

In Salmonella typhimurium, Clowes (1958) isolated series of mutants which were similar to those of E.coli except that one mutant although able to utilize sulphate was unable to utilize thiosulphate. Further the addition of thiosulphate strongly inhibited the uptake of (^{35}S) sulphate, as it had in E.coli (Roberts et al., 1955), so Clowes concluded that thiosulphate was involved in one or two pathways of sulphate reduction, the other of which bypassed thiosulphate in the sulphiteless mutant unable to use thiosulphate, as was also suggested by Hockenhull (1948) for Aspergillus nidulans. The presence of a thiosulphateless mutant in E.coli also supports thiosulphate as an intermediate in sulphate reduction by this bacteria. However Dreyfuss and Monty (1962) found that a thiosulphateless mutant of Salmonella typhimurium, when growing on thiosulphate, released sulphite from the outer S atom into the medium. They suggested that thiosulphate was reductively dismutated into sulphite and sulphide as was reported in extracts of yeast by Kaji and McElroy (1959) when glutathione was added.

$$SSO_3'' \longrightarrow S'' + SO_3'' \qquad (2)$$

Further Leinweber and Monty (1963) found that the assimilation of one of the S atoms of thiosulphate in Salmonella depended upon the presence of sulphite reductase, so in this bacterium thiosulphate was cleaved to sulphite and sulphide. Therefore thiosulphate was equivalent to sulphide as it gave rise to this, in the thiosulphateless mutants.

Extracts of Salmonella cells failed to catalyze reduction of thiosulphate unless reduced glutathione was present and in the latter case the reduction occurred regardless of the nutritional evidence, so the enzymic basis of thiosulphate reduction could not be established. Leinweber and Monty (1963) found that cysteine repressed and sulphide inhibited the assimilation of thiosulphate by whole cells.

They suggested from this that the failure to observe thiosulphate reduction in extracts was due to strong product inhibition by sulphide. Dreyfuss and Monty (1963) found that reduction of 5'-phosphoadenosine-3'-phosphosulphate (PAPS) (an intermediate in the reduction of sulphate by yeast), sulphite and thiosulphate (the latter by whole cells) was repressed to the maximum extent by cysteine and the least by djenkolic acid ($HOOCCH(NH_2)CH_2-S-CH_2-S-CH_2CH(NH_2)COOH$). It therefore seems that thiosulphate, although not directly involved in sulphate reduction is of some importance to Salmonella.

Certain of the Salmonella mutants examined by Dreyfuss and Monty (1962) were unable to grow on thiosulphate or sulphate, although they grew on sulphite, as was also noted by Clowes. These mutants also failed to accumulate sulphite. Examination of the extracts for thiosulphate reduction would have established nothing, but a thiosulphateless mutant which also carried a mutation in the region responsible for sulphite reduction ('Cd' type) failed to accumulate sulphite from thiosulphate as the Cd mutant alone would have done. Therefore the mutant able to grow on sulphite but not on thiosulphate was either unable to transport or unable to metabolize thiosulphate and the failure to grow on thiosulphate does not indicate an alternative pathway for sulphate reduction as had been suggested by Clowes.

For Bacillus subtilis, thiosulphate was shown to be a strong inhibitor of sulphate uptake by Villarejo and Westley (1966) but they also showed that sulphide at non-toxic concentrations reduced the uptake of (^{35}S) thiosulphate into cells. From the conclusions reached for Salmonella and Proteus it is most reasonable to explain this result and existence of a thiosulphateless mutant of E. coli by the same mechanism.

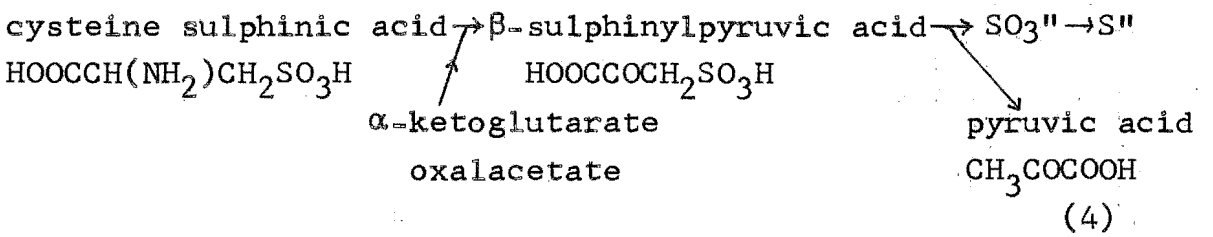
Now the sulphiteless mutants of Salmonella typhimurium also utilized cysteine sulphinic acid ($HOOCH(NH_2)CH_2SO_2H$) (CSA), (Clowes, 1958). Cobey and Handler (1956) were unable to find any CSA synthesis from sulphite by extracts of E.coli,

Leinweber and Monty (1961) investigated CSA metabolism by *E. coli* and found that CSA increased the formation of sulphide by cells and extracts of cells. The increase brought about by the addition of sulphite was not augmented by CSA and the stimulation by cysteine of sulphide production was exactly additive to that brought about by CSA in both whole cells and extracts. Hence sulphite was an obligatory intermediate in the metabolism of CSA.

cysteine sulphinic acid $\longrightarrow$ sulphite $\longrightarrow$ sulphide (3)
$HOOCCH(NH_2)CH_2SO_2H$ $SO_3"$ S"

Kearney and Singer (1953) had earlier found that resting cells of *Proteus vulgaris* oxidized CSA predominantly by a transaminative pathway. Extracts of Salmonella reacted in the same way as did those from *E. coli* (Leinweber and Monty, 1961).

Leinweber and Monty (1962) later separated the extracts of *E. coli* into two fractions; one reducing sulphite with NADPH[a], similar to that isolated by Mager (1960) and the other producing sulphite from CSA by a transaminase reaction requiring pyridoxal phosphate, which was stimulated by α-ketoglutarate and presumably yielded β-sulphinyl pyruvate and the latter then broke down to pyruvate and sulphite. The fraction reducing sulphite produced sulphide.



(4)

It thus seems firmly established that cysteine sulphinic acid is not an intermediate in sulphate reduction by *E. coli*. The results of Leinweber and Monty for Salmonella (1961) as well as the observation of Dreyfuss and Monty (1962) that mutants of Salmonella whose extracts failed to reduce sulphite

[a] nicotinamide adeninedinucleotide phosphate (reduced form)

were unable to grow on either sulphite or CSA strongly support a similar position of CSA in this bacterium as well.

An enzyme reducing sulphite and hydroxylamine, and requiring NADPH was isolated from E. coli by Mager (1960). This enzyme was later reported by Leinweber and Monty (1962) to produce sulphide. Gilboa-Garber and Mager (1966) purified the enzyme 400 fold but the hydroxylamine reducing activity was not dissociated from the sulphite reducing activity. Pasternak, Ellis, Jones-Mortimer and Chrichton (1965) found that sulphite reductase of E.coli and B.subtilis was repressed by growth on cysteine and not by growth on sulphite. Thus it seems clear that, although the enzyme reduced hydroxylamine, its physiological function was the reduction of sulphite. The results of Pasternak, et al. (1965) also suggested that the reduction of sulphate to sulphite in E.coli and B.subtilis is similar to that in other microorganisms especially yeast as discussed by Wilson (1962). These authors isolated an enzyme reducing PAPS to sulphite with NADPH which was repressed by growth on cysteine, as well as by growth on sulphite. Wheldrake and Pasternak (1965) found that ATP sulphurylase and APS kinase of E.coli and B.subtilis are each repressed by growth of the bacteria with cysteine, whereas ATP sulphurylase of Desulphovibrio desulphuricans, in which sulphate reduction is linked to the energy supply of the organism, was not repressed by growth in the presence of sulphite or cysteine.

(5)

$$SO_4^{=} + ATP \xrightarrow{\text{ATP sulphurylase}} \text{Adenosine-5'-phosphosulphate (APS)}$$

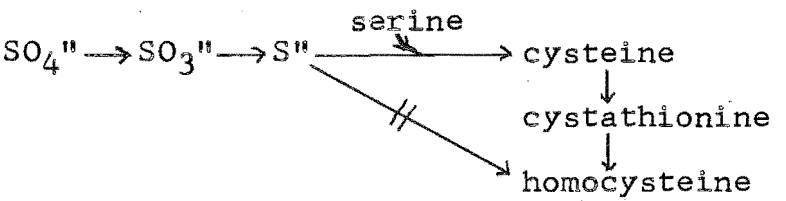
$$\text{APS} \xrightarrow[\text{APS kinase}]{\text{ATP}} \text{5'-phosphoadenosine-5'-phosphosulphate (PAPS)} \quad (6)$$

For Salmonella typhimurium, Dreyfuss and Monty (1962) reported that extracts of wild type cells catalyzed the formation of sulphide from either sulphate, APS, PAPS or sulphite. Mutants which were unable to grow on sulphate but

able to on sulphite, lacked either ATP sulphurylase, APS kinase or PAPS reductase. Mutants which were unable to grow on sulphite lacked sulphite reductase. Sulphate reduction thus appears to be similar to that in E.coli and yeast.

$$SO_4 \longrightarrow APS \longrightarrow PAPS \xrightarrow{\text{PAPS reductase}} SO_3'' \longrightarrow S'' \qquad (7)$$

Ellis (1966) isolated the N-ethylmaleimide (NEM) derivatives of sulphite and sulphide from cells of E.coli, fed (^{35}S) sulphate for a few seconds, and found that the sulphite derivative was labelled first. The incubation of the cells with cysteine along with the (^{35}S) sulphate resulted in NEM-cysteine, an unknown compound, NEM-sulphite and NEM-sulphide. Cysteine appeared to be the amino acid through which the sulphur atom is transferred from sulphate to homocysteine, rather than directly as suggested by Wiebers and Garner (1960) for Neurospora.



(8)

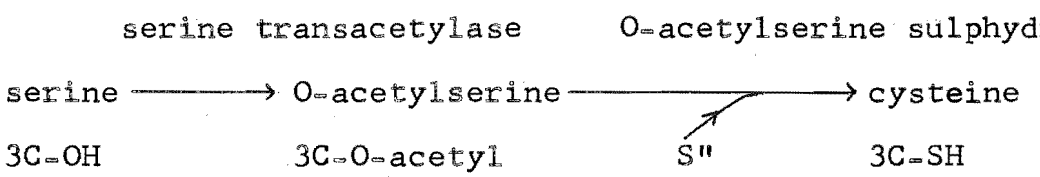
NEM does not form a stable derivate with thiosulphate (Ellis, 1966; Trudinger, 1965) so that the role of thiosulphate in sulphite reduction could not be established by this technique.

The importance of sulphite and sulphide in the assimilation of sulphate by bacteria thus seems firmly established. The two alternate pathways involving thiosulphate or cysteine sulphinic acid seem most unlikely on present evidence. The position of sulphite and sulphide as obligatory intermediates in sulphate reduction is favoured by the isolation of the appropriate mutants and enzymes as well as by isotope competition studies and the

isolation of these compounds themselves, in conditions under which they would most likely have formed from sulphate. The intermediate role of sulphide in cysteine synthesis is further supported by the synthesis of cysteine from sulphide and a derivative of serine, as described below.

Schlossmann and Lynen (1957) and Schlossmann, Bruggemann and Lynen (1962) isolated a serine sulphydrase from yeast (or cysteine synthase) which was pyridoxal phospate dependent and added sulphide to serine to form cysteine. Bruggemann, Schlossmann, Merkenschlager and Waldschmidt (1962) reported the presence of this enzyme in several bacteria including E.coli, two fungi, spinach, chicken and rat tissues. However Kredich and Tomkins (1966) isolated from Salmonella and E.coli an enzyme forming cysteine from O-acetyl-L-serine and sulphide. They reported two enzymic activities; the first catalyzed the formation of O-acetylserine from serine and the second the formation of cysteine.

(9)



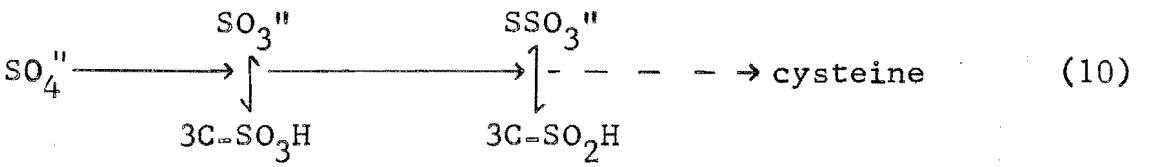
The former activity was purified about 1000 fold and had a spectrum similar to that for other pyridoxal-containing enzymes. O-acetylserine sulphydrase activity was repressed in Salmonella grown on cysteine and derepressed when grown on L-djenkolic acid. Pasternak et al. (1965) reported in E.coli that the enzymes catalyzing sulphate activation and reduction, and sulphite reductase were repressed by growth on cysteine. Dreyfuss and Monty (1962) had reported a similar pattern of repression by cysteine and derepression by djenkolic acid for the enzymic activities reducing PAPS and sulphite in Salmonella.

Kredich and Tomkins (1966) also reported that cys-E mutants of Salmonella (cys- mutants are cysteineless mutants of which a type is denoted by the E) contained undetectable

levels of serine transacetylase and had variable levels of O-acetylserine. For the latter enzyme L-serine and several other compounds similar to O-acetylserine did not substitute for O-acetylserine. From this it seems that the serine sulphydrase activity reported by Bruggemann et al. (1962) could either involve an O-acetyl derivative of serine or is unlikely to be important physiologically, especially as it was present in the chick and rat in which no marked synthesis of cysteine from sulphate has been shown.

The assimilation of sulphate by Fungi

From studies on mutants of Neurospora crassa, Horowitz (1950) suggested a pathway of sulphate reduction involving cysteine sulphinic acid (CSA) in equilibrium with thiosulphate.



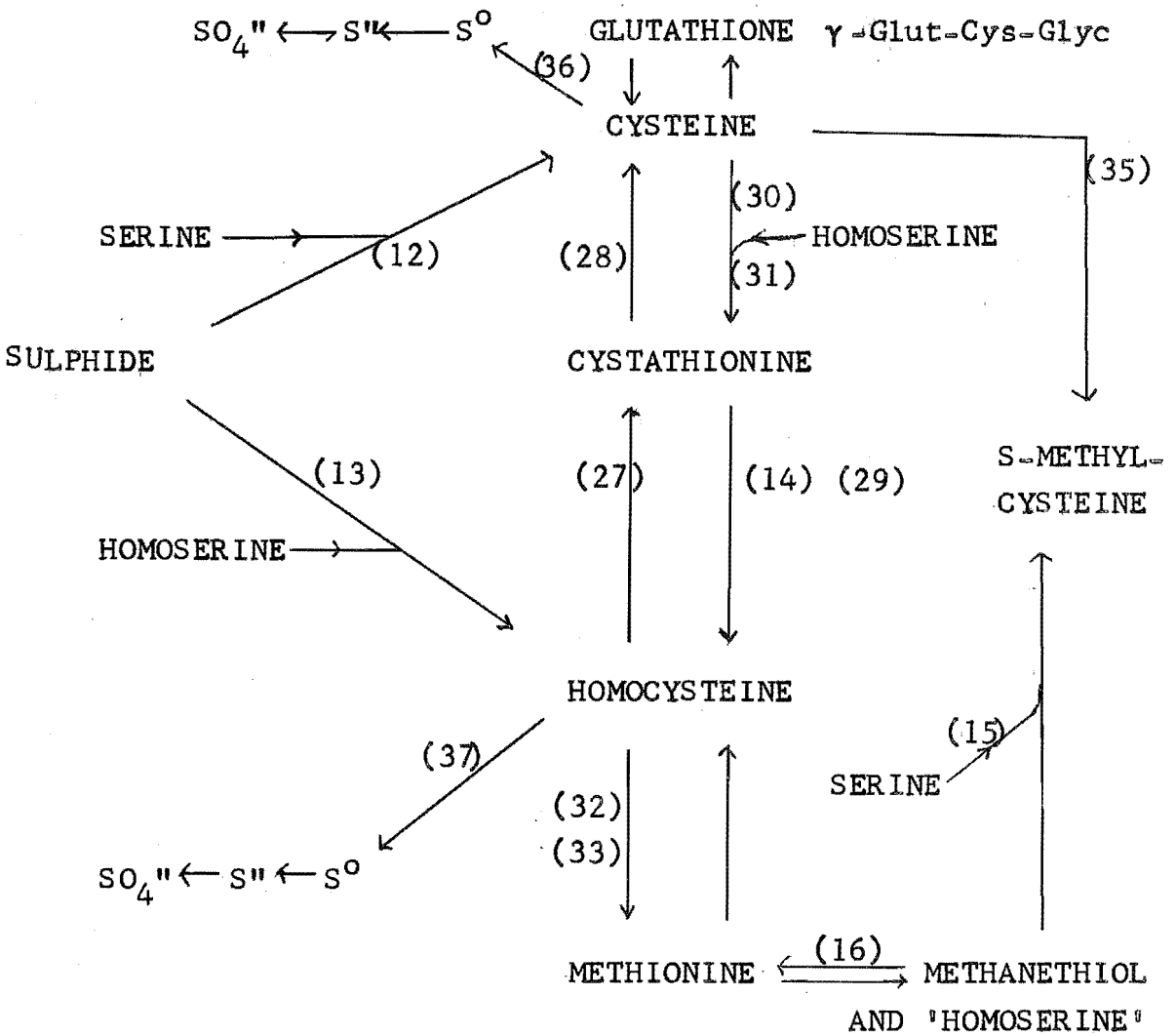
cysteic acid cysteine sulphinic acid (10)

Leinweber and Monty (1965) found that mycelia of Neurospora rapidly released sulphite into the medium from CSA and that extracts of Neurospora similarly formed sulphite in the presence of either α-ketoglutarate or oxalacetate. Pyridoxal phosphate only slightly stimulated this reaction in contrast to that in E.coli. CSA was therefore degraded to sulphite, just as for E.coli and Salmonella, before contributing sulphur to Neurospora. Mycelial extracts reduced sulphite to sulphide, requiring flavin adenine dinucleotide (FAD) and NADPH, which are slightly different requirements from those of E.coli. No significant formation of sulphide from thiosulphate could be detected, so sulphite and sulphide appear to be intermediates in sulphate reduction by Neurospora.

Wilson (1962) extensively reviewed sulphate reduction to sulphite in yeast. The sulphate ion is first activated by the formation of adenosine-phosphosulphate (APS); this reaction is catalyzed by adenosine triphosphate (ATP) sulphurylase.

$$\text{sulphate} + \text{ATP} \xrightarrow{\text{ATP sulphurylase}} \text{adenosine-5'-phosphosulphate (APS)} \qquad (5)$$

An additional phosphate group is added to APS by APS kinase to produce 3'-phospho-adenosine-5'-phosphosulphate (PAPS).



Several possible reactions involved in the interconversion of cysteine and methionine.

The numbers refer to equations in the text.

$$\text{APS} + \text{ATP} \xrightarrow{\text{APS kinase}} \text{3'-phosphoadenosine-5'-phosphosulphate (PAPS)} \quad (6)$$

The latter is reduced by PAPS reductase to give sulphite. As well as PAPS reductase, the reaction involves a heat stable protein with a disulphide group (Fraction C) whose reduction by nicotinamide adeninedinucleotide phosphate (NADPH) is catalyzed by enzyme A. The reduced fraction C is involved in the reduction of PAPS.

$$\text{PAPS} \xrightarrow[\text{NADPH}]{\text{PAPS reductase, Enzyme A, Fraction C}} \text{sulphite} + \text{PAP} \quad (11)$$

Ragland (1959) found that one of two sulphiteless mutants of Neurospora lacked ATP sulphurylase and the other had increased levels of that enzyme. From the former he considered ATP sulphurylase was important in sulphate assimilation and explained the latter as lacking either APS kinase or PAPS reductase. Neurospora appeared to reduce sulphate to sulphite in a similar manner to yeast.

Leinweber and Monty (1965) also reported the synthesis of cysteine from serine and sulphide with cell free extracts of Neurospora and that pyridoxal phosphate stimulated this synthesis. Thiosulphate and sulphite were unable to replace sulphide and of the carbon chains supplied, only L-serine was effective, although homoserine had one-sixteenth of the activity of serine.

More recently Wiebers and Garner (1967) reported in Neurospora the presence of an enzyme or enzymes adding sulphide to homoserine as well as to serine.

$$S'' + \text{serine} \xrightarrow{\text{cysteine synthetase}} \text{cysteine} \quad (12)$$

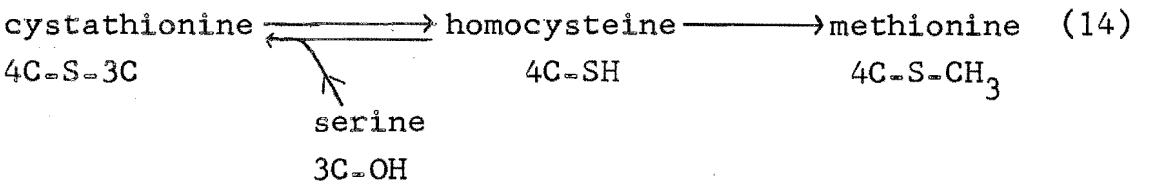
$$S'' + \text{homoserine} \xrightarrow{\text{homocysteine synthetase}} \text{homocysteine} \qquad (13)$$

In a crude extract (slightly purified) of Neurospora, the homocysteine synthetase activity was a quarter of that of the cysteine synthetase. However, although this activity was proportionately greater than the homocysteine synthetase activity reported by Leinweber and Monty, the results of the two groups are not directly comparable as different extraction conditions were used to isolate the enzymes.

Leinweber and Monty, for instance, froze the mycelium at -15°C. and ground it with sand at pH 8.0, in 0.04 M phosphate buffer containing 0.005 M EDTA. Wiebers and Garner broke the cells in cold tris buffer pH 8.2 with glass beads at 4°C. Both preparations were centrifuged and the soluble fraction taken.

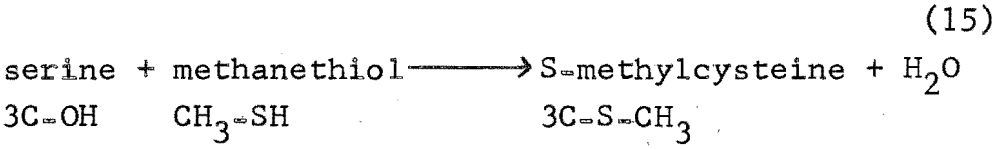
Wiebers and Garner found that cysteine synthetase and homocysteine synthetase activities were repressed in homocysteineless and cystathionineless mutants when these were grown on methionine. However, cysteine synthetase was repressed by growth of the cells on cysteine, as was homocysteine synthetase by growth on homocysteine, only as much as the activities of these enzymes in wild type cells. Growth of wild type on homocysteine stimulated cysteine synthetase whereas wild type and cysteineless mutants grown on cysteine contained similar levels of homocysteine synthetase. These enzymic activities were non competitively inhibited by methionine. Serine and homoserine inhibited the other's reaction and cysteine and homocysteine inhibited both their own and the other's synthesis. From this and their earlier work (1960, 1964, 1966) Wiebers and Garner concluded that cysteine and homocysteine were synthesized independently and that their synthesis was controlled by the several intermediates involving cystathionine as a key compound.

In 1960 Wiebers and Garner reported that the (^{35}S) and (^{14}C) atoms of labelled cystathionine fed to a cystathionineless mutant for six days were found to different extents in methionine. The sulphur atom was incorporated to a greater extent than the carbon atoms. They concluded that the following reaction was not the only pathway of methionine synthesis:



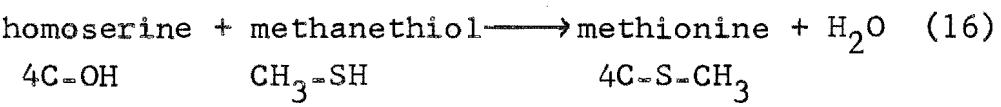
(14)

Ragland and Liverman (1956) found that two methionineless mutants of Neurospora grew on S-methylcysteine (CH_3-S-CH_2CH-(NH_2)COOH). Hence methionine was synthesized through a pathway not involving homocysteine. Wolf et al. (1956) reported that yeast extracts catalyzed the synthesis of S-methylcysteine from serine and methanethiol.



(15)

Possibly Neurospora synthesizes methionine by an analogous reaction:



(16)

after S-methylcysteine is degraded to methanethiol. If this pathway is involved in methionine biosynthesis from sulphate then it would have to be blocked at the same time as the pathway through cystathionine. These reactions suggest a possible explanation for the differential incorporation of labelled atoms. If methionine is degraded by a reversible reaction to methanethiol:

$$4C\text{-}S\text{-}CH_3 \longleftrightarrow X + Y + CH_3\text{-}SH \qquad (17)$$

and the compound X is present in a pool utilized for the synthesis of some other compound, then the labelled carbon atoms of methionine would be replaced by the unlabelled carbons of X even though no synthesis of methionine was occurring by this route. Wiebers and Garner (1964) found that (^{35}S) and (^{14}C) labelled cystathionine, added to the medium in increments related to the amount of growth, were incorporated equally well (although rather poorly).

For Neurospora present evidence indicates that sulphate is assimilated through sulphite and sulphide into cysteine and possibly through sulphide also into homocysteine. It is not clear yet whether the sulphide incorporation involves the formation of O-acetyl or similar derivatives of serine and homoserine as has been reported for serine in E.coli, and for homoserine in cystathionine synthesis by E.coli (Rowbury, 1964).

To study the sulphur metabolism of Aspergillus nidulans, Hockenhull (1949) examined a number of mutants. He characterized, among others, sulphateless, sulphiteless and thiosulphateless mutants. The last could be explained in the same way as were the Salmonella thiosulphateless mutants but for the nonutilization of sulphide by certain of the thiosulphateless mutants. Hockenhull considered that the simplest hypothesis to explain this, in view of the fact that cysteineless mutants also grew on cysteine-S-sulphonate, ($HOOCCH(NH_2)CH_2SSO_3H$) was an incorporation of thiosulphate into cysteine-S-sulphonate by combination with serine.

$$SO_4'' \longrightarrow SO_3'' \longrightarrow \underset{\text{thiosulphate}}{SSO_3''} \xrightarrow{\text{serine}} \underset{\text{cysteine-S-sulphonate}}{3C\text{-}SSO_3'} \qquad (18)$$

Shepherd (1956) found that acetone powder extracts of Aspergillus nidulans synthesized cysteine from thiosulphate. This was stimulated by the addition of serine but not by pyridoxal phosphate.

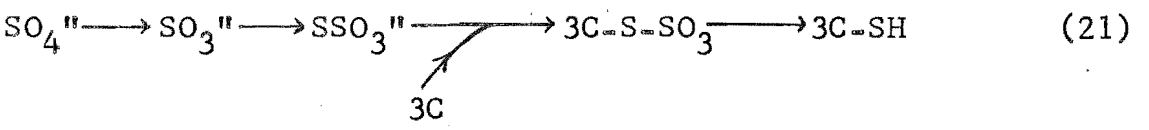
$$SSO_3'' + \underset{3C\text{-}OH}{\text{serine}} \longrightarrow \underset{3C\text{-}SH}{\text{cysteine}} \qquad (19)$$

Further Nakamura and Sato (1963a) found that extracts of Aspergillus synthesized cysteine-S-sulphonate from thiosulphate and serine. ATP, MgCl and Pyridoxal phosphate were essential cofactors for the reaction. This result is not necessarily inconsistent with Shepherd's as their preparation was dialyzed and his was not. Nakamura and Sato also found that a mutant requring cysteine-S-sulphonate or cysteine for growth did not synthesize S-sulphocysteine in the complete reaction system, whereas a mutant able to grow on cysteine and unable to grow on cysteine-S-sulphonate did. In (1963b) they reported on a mutant unable to grow on cysteine-S-sulphonate and found that it accumulated (^{35}S) cysteine-S-sulphonate which was labelled in both S atoms, when fed (^{35}S) sulphate. They also identified (^{35}S) cysteine-S-sulphonate in the wild type and failed to find it in either a thiosulphateless, sulphiteless or a cysteine-S-sulphonateless mutant. Since S-sulphocysteine was labelled in both atoms, a nonenzymic interchange between the sulphonate group and sulphite was ruled out (Kolthoff and Stricks, 1951) as an explanation of the labelling.

$$R\text{-}S\text{-}\overset{*}{S}O_3' + R'\text{-}S' \longleftrightarrow R\text{-}S\text{-}S\text{-}R' + \overset{*}{S}O_3'' \qquad (20)$$

Nakamura and Sato therefore concluded that cysteine-S-sulphonate was formed by a combination of thiosulphate with a three carbon acceptor, probably serine and that their results were in accord with cysteine-S-sulphonate being an

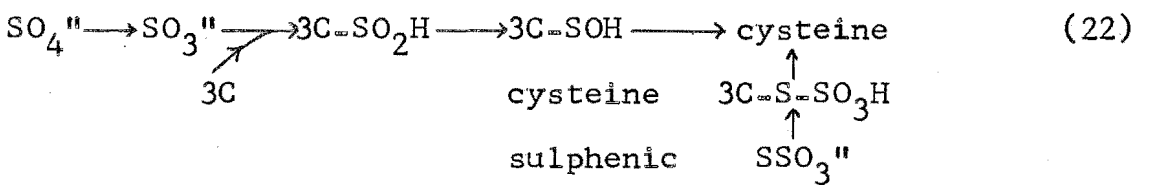
obligatory intermediate in the synthesis of cysteine.



(21)

Yoshimoto, Nakamura and Sato (1961) isolated a sulphite reductase from Asperigillus nidulans which formed sulphide. Although this suggests that the pathway involving thiosulphate may not be the only one, unless sulphide is oxidized to thiosulphate, the reductase purified 100 times coupled only with reduced methyl viologen (MVH) or hydrogenase and did not accept either NADH or NADPH as reducing agents, so that its physiological role remains doubtful.

Shepherd (1956) also found that Aspergillus extracts catalyzed the formation of cysteine sulphinic acid from (^{35}S) sulphite when pyridoxal phosphate was present. The addition of glutamate and pyruvate stimulated the reaction, so it appeared that a transaminase was involved. Since he also found a thiosulphateless mutant able to grow on CSA, he concluded that sulphate was assimilated through two pathways, the one involving CSA and the other cysteine-S-sulphonate.



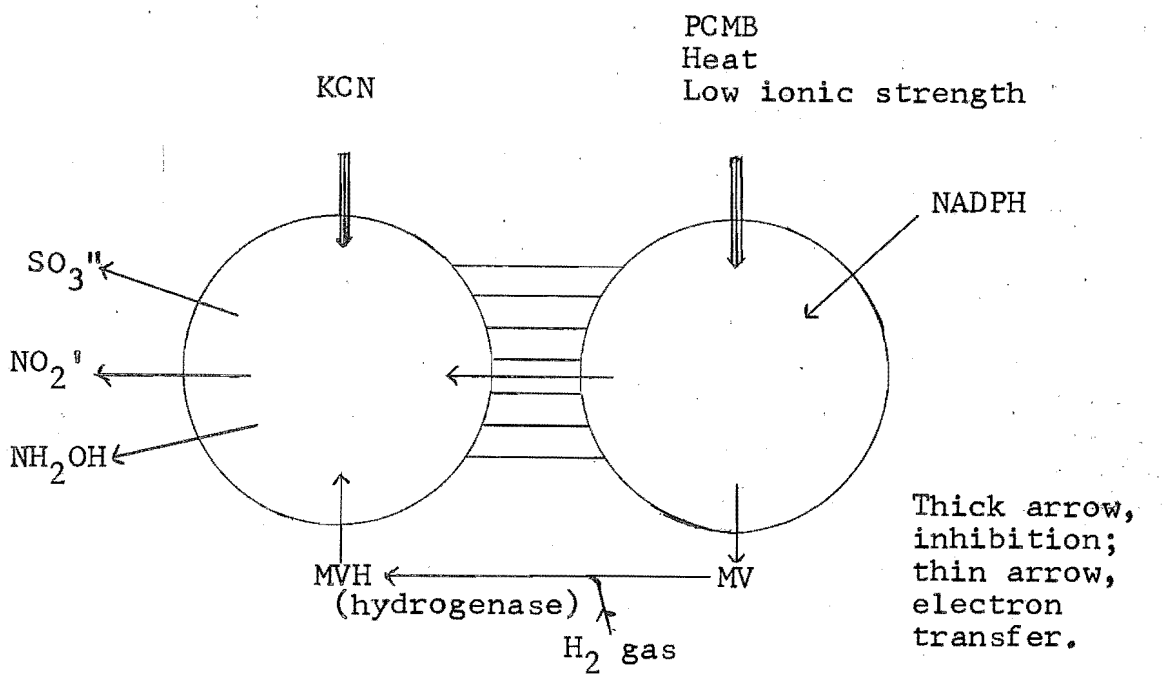
(22)

In this case mutants failing to utilize sulphite would have to be blocked in both pathways simultaneously. Chapeville and Fromageot (1954) found that an acetone powder of rabbit kidney extracts formed CSA from sulphite and this organ has not been reported to synthesize cysteine from sulphate. From this, the synthesis of cysteine sulphinic acid reported by Shepherd might not be on the pathway of cysteine formation in which case the mutant able to use cysteine

sulphinic acid but not sulphite must produce sulphite from cysteine sulphinic acid, in the way that Salmonella does, and also fail to transport sulphite into the mycelium.

In contrast to other microorganisms it appears in Aspergillus that the pathway of sulphate assimilation involves thiosulphate and cysteine-S-sulphonate.

For yeasts a sulphite reductase coupling with NADPH was reported by Lezius (1959) to produce sulphide. Naiki (1965) further examined the properties of a sulphite reductase from Saccharomyces cerevisiae. He found that extracts from several mutants oxidized MVH if they possessed NADPH-sulphite reductase activity but some mutants contained MVH activity only. After the enzyme was purified about 200 fold the ratio of MVH to NADPH activity remained more or less constant. However the NADPH activity alone was lost if the enzyme was exposed to heat, low ionic strengths or p-chloromercuribenzoate (PCMB). On the other hand cyanide affected both equally. From this Naiki proposed a two part model for sulphite reductase in which the electrons are donated by NADPH to the first part and these then flow to the second part which is also able to accept electrons from MVH. The enzyme also reduced hydroxylamine and nitrite.



Wainwright (1962a) reported that extracts catalyzing sulphite reduction could be split into six fractions, two of which were identical with two fractions of the sulphate-reducing system examined by Wilson, Asahi and Bandurski (1961). Not all of the fractions were needed for sulphite reduction but in that case NADH could replace NADPH equally well.

Wainwright (1962b) also reported the reduction of sulphite to sulphide by pantothenate-deficient yeast cells and extracts and this was inhibited by growth of cells on methionine. However Okuda and Uemura (1965) divided extracts of pantothenate sufficient and deficient yeast cells into three fractions by ammonium sulphate precipitation and found that pantothenate-sufficient cells have most of the reductase activity in one fraction (R3) whereas pantothenate-deficient cells have most of the activity in another fraction (R1) and the activity of this last fraction was suppressed by growth of cells on methionine. Since the properties of the R3 fraction were similar to those of the sulphite reductase isolated by Lezius (1959), the authors concluded that growth of the cells on pantothenate-deficient media caused the appearance of a new sulphite reductase.

Naiki (1961) reported that yeast strains cultured in a copper-containing medium produced much hydrogen sulphide in the lag phase. Kikuchi (1965a) showed that copper resistant strains obtained by culturing in copper medium produced more hydrogen sulphide from sulphate, sulphite or thiosulphate than did the parent strains. However in a study reported in 1965b of sulphide production from sulphite by copper-adapted strains, grown both in copper-containing medium and copper-free medium, Kikuchi found that they produced more sulphide than did the parent strains but they were also more sensitive to higher concentrations of sulphite and to salicylaldoxime. He suggested from this that the pathway from sulphite to sulphide differed after exposure of the cells to copper.

Hilz, Kittler and Knape (1959) found that thiosulphate inhibited formation of sulphite and sulphide from sulphate by yeast extracts but only by competitive inhibition of sulphate activation. Also Kaji and McElroy (1959) found that a cell free system from yeast reduced thiosulphate to sulphite and suphide in the presence of reduced glutathione. Hence it seems most likely that sulphite and sulphide are directly coupled intermediates in the reduction of sulphate, as in the bacteria and Neurospora.

Schlossmann and Lynen (1957) found that yeast extracts synthesized cysteine from serine and sulphide and that the reaction was stimulated by pyridoxal phosphate. Hence it is most likely that sulphate is reduced in yeast to sulphite and then to sulphide before incorporation into cysteine.

Sulphate reduction in animals

Several workers have reported the formation of cystine in animals but is difficult to assess the contribution of microorganisms in the gut. For instance, Waldschmidt (1962) found that radioactive cystine was formed from (^{35}S) sulphate injected into the rat, but she concluded that the sulphate was transported to the gut where microorganisms reduced the sulphate and the product was then reabsorbed into the rat. Machlin, Pearson, Denton and Bird (1953) reported that eggs of laying hens injected with (^{35}S) sulphate contained radioactive cystine but not radioactive methionine and concluded from this that microbial transformation was not involved.

Johnston, Harvey and Bowers (1966) found that fertile eggs injected with sterile (^{35}S) sulphate labelled soluble cystine. Infertile eggs contained no radioactive cystine. The authors found that the specific activity of cystine declined from the tenth day of incubation, after injecting the isotope on the first day, to the day of hatching; although the per cent of the dose found in cystine increased from about one to about two at the end of the period. Since carrier free sulphate was used, the quantity of cystine synthesized from the sulphate in the egg was a small proportion of the sulphate present.

On the other hand Machlin, Pearson and Denton (1955) found that about 65% of the (^{35}S) sulphate injected into fertile eggs was recovered in taurine in the day old chick. They were unable to find any radioactive cystine, methionine or cysteic acid and so concluded that the taurine was not formed through cysteine. Chapeville and Fromageot (1957) investigated how taurine was formed in the chicken embryo and found, both in the organism and in extracts, that sulphate was reduced to sulphite which was then incorporated into cysteic acid. The reduction was catalysed only by the vitelline chamber and the formation of cysteic acid only by the yolk. Cysteic acid was then converted into taurine.

The enzymic incorporation of radioactivity into cysteic acid was not reduced by the addition of cysteine sulphinic acid so the latter was almost certainly not an intermediate in the synthesis of cysteic acid. Cysteic acid both in the egg and in a homogenate of the embryo or the yolk was decarboxylated to form taurine.

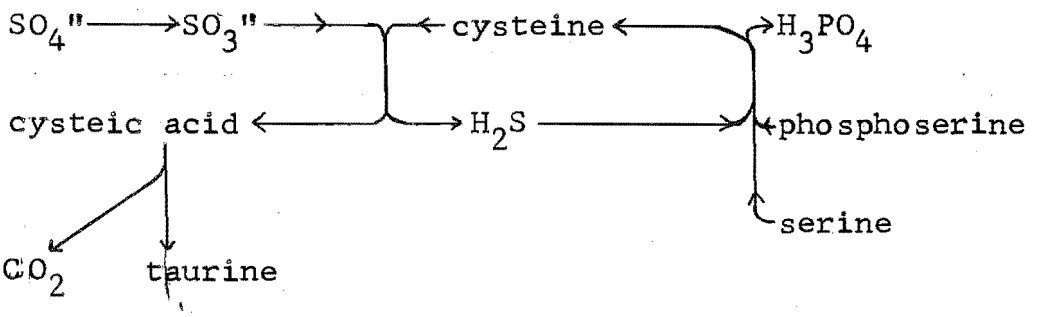
$$SO_4" \longrightarrow SO_3" \longrightarrow \underset{\text{cysteic acid}}{HOOCCH(NH_2)CH_2SO_3H} \longrightarrow \underset{\text{taurine}}{HCH(NH_2)CH_2SO_3H} \qquad (23)$$

From an examination of the time of appearance of taurine and cysteic acid in extracts and their relative specific activities, Chapeville and Fromageot (1957) concluded that taurine was not formed directly from sulphite. They found that the synthesis of cysteic acid was stimulated by cysteine and inhibited by pyruvic acid and α-ketoglutarate, and that hydrogen sulphide was formed at the same time. They concluded that the four carbon skeleton for the reaction with sulphite was derived from cysteine. In 1961 Chapeville and Fromageot reported that a pyridoxal phosphate-requiring enzyme catalyzed the replacement of the sulphydryl on the β-carbon of cysteine by sulphide, sulphite or cysteine itself.

$$3C\text{-}SH + SO_3" \xrightarrow{\text{cysteine } H_2S\text{-lyase}} 3C\text{-}SO_3H + S" \qquad (24)$$

The enzyme was isolated from the yolk of egg and was found to be free from iron, copper and magnesium.

Sentenac, Chapeville and Fromageot (1963) reported that a purified extract from the vitelline chamber of a seventeen day old chick catalyzed the synthesis of cysteine from (^{35}S) sulphide and phosphoserine. Serine was ineffective and the enzymic activity was restricted to the vitelline chamber. Since cysteine H_2S-layse is also present in the vitelline chamber, Sentenac et al. suggested that the synthesis of taurine involved the following cycle:



(25)

This pathway is presumably unable to synthesize cysteine since the reduction of sulphite to sulphide is lacking.

Miraglia, Martin, Spaeth and Patrick (1966) found in young chicks that the radioactivity of (3-^{14}C) cysteine, (U-^{14}C) alanine, (3-^{14}C) serine, which are three-carbon compounds, was incorporated into taurine as was that of (1,2-^{14}C) ethanolamine and (1-^{14}C) glycine, which are two-carbon compounds. Therefore it seems that although cysteine contributes to the carbon skeleton of taurine this was not a major source since so many compounds can so contribute.

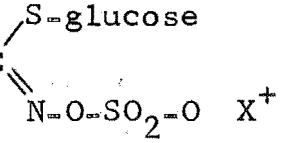
The addition of serine, glycine, ethanolamine as well as sodium isethionate ($HOCH_2CH_2SO_3H$), cysteine and methionine to a low sulphur amino acid diet, increased the incorporation of (^{35}S) sulphate into taurine. The stimulation of (^{35}S) sulphate incorporation into liver-taurine by the addition of sulphate and the reduction of total taurine by dietary cysteine in the chick reported by Martin, Miraglia, Spaeth and Patrick (1966) was consistent with this.

Martin et al. (1966) also found radioactive compounds at the Rf's of cystine, methionine, isethionic acid, cysteamine and hypotaurine in extracts of chick fed (^{35}S) sulphate. They suggested from this that these compounds might be intermediates in the synthesis of cysteine.

Sulphate assimilation in higher plants

Thomas, Hendricks, Bryner and Hill (1944) found that wheat, corn and barley plants formed radioactive organic compounds from (^{35}S) sulphate and from (^{35}S) sulphur dioxide. However when sulphur dioxide was fed, sulphate as well as the organic compounds became labelled, so (^{35}S) sulphite could not be established as an intermediate of sulphate assimilation.

Tobacco leaves were found by Fromageot and Perez-Milan (1959) to form (^{35}S) sulphite from (^{35}S) sulphate. The authors fed the excised leaves through the petiole with a solution of (^{35}S) sulphate containing sodium sulphite to trap the (^{35}S) sulphite formed. Illuminated leaves were found to form more (^{35}S) sulphite than leaves kept in the dark. Asahi (1960), Asahi and Minamikawa (1960), Kawashima and Asahi (1961) found that mung bean leaves formed both (^{35}S) sulphite and (^{35}S) sulphide from (^{35}S) sulphate when vacuum infiltrated with a solution of the radioisotope. The incorporation of (^{35}S) sulphite into organic compounds was not affected by the simultaneous addition of sulphate although the incorporation of (^{35}S) sulphate was reduced by unlabelled sulphite. Since (^{35}S) sulphide was also formed from (^{35}S) sulphite the authors considered that sulphite and sulphide were intermediates of sulphate reduction. When (^{35}S) sulphite was infiltrated into the leaves, cysteine sulphinic acid was labelled as well as sulphide but the significance of this is not clear.

In horseradish, Chisholm and Wetter (1964) found that methionine was an intermediate in the synthesis of sinigrin - a thioglucoside,  where for sinigrin R is CH_2= CH-CH_2-O. After reaction of sinigrin with myrosinase, (a hydrolytic enzyme), sulphate, glucose and allyl isothiocyanate (CH_2=CH-CH_2NCS) are the products. The (^{14}C) methionine contributed ^{14}C carbon atoms to the allyl isothiocyanate with little randomization. The sulphur atom of the allyl isothiocyanate was found by Wetter (1964) to be labelled by (^{35}S) methionine also. Further (^{35}S) sulphate

labelled both the sulphate ester of sinigrin and the isothiocyanate group whereas (^{35}S) methionine labelled the former hardly at all.

When labelled sulphide or thiosulphate was fed to horseradish leaves, the ratio of radioactivity in the two groups (sulphate ester and isothiocyanate) was similar to that when labelled sulphate was fed. This indicated that these compounds were incorporated through sulphate and not as intermediates in their own right. Also the incorporation of the inner atom of thiosulphate (i.e. $^{35}SSO_3''$) was much superior to that of the outer atom. Hence the metabolism of thiosulphate appears to be similar to that in E. coli, Salmonella and Neurospora, in which reductive cleavage splits thiosulphate into sulphite and sulphide. After this, horseradish appears to oxidize these two to sulphate whereas the microorganisms incorporate them directly.

Weigl and Ziegler (1962) fed (^{35}S) sulphite to spinach leaves. (^{35}S) sulphate was quickly formed and after two hours a considerable quantity of the radioactivity was present in sulphate while cysteine and glutathione were also radioactive.

These results are at variance with those of Asahi et al. but they can be reconciled if the compounds fed by Wetter and by Weigl and Ziegler were oxidized without reaching the sites of sulphate reduction so that their metabolism was not that of suphite and sulphide involved in sulphate reduction. Since it appears likely (as will be discussed later) that the intermediate sulphite is not free during sulphate reduction, this possibility is quite likely. On the whole then these results support the intermediate role of sulphite and sulphide in sulphate assimilation by higher plants and this is similar to that in bacteria, Neurospora and yeast.

That sulphate reduction to sulphite in higher plants was also similar to the process in yeast was suggested by Squires (1962) finding a sulphate activation enzyme in acetone powders of spinach leaves. Asahi (1964) found that chloroplasts from spinach leaves, suspended in Tris buffer pH 7.5 plus ATP and magnesium ions, produced active sulphate

(APS) from sulphate.

$$ATP + SO_4 \xrightarrow{\text{ATP sulphurylase}} APS + \text{pyrophosphate} \qquad (5)$$

The addition of a sulphate activation system from yeast and Fraction C allowed the formation of (^{35}S) sulphite. Illumination of the chloroplasts was also necessary and the fraction C-SS (Fraction C, called fraction C-SS by Asahi), when added by itself, was reduced by the illuminated chloroplasts. In addition to the reduction of fraction C-SS, a C-SS-like substance in the chloroplast was also reduced in the light and this was partially purified and found to be reduced by NADPH and C-SS reductase (enzyme A of Yeast). These results suggested that reduction of sulphate to sulphite was through APS and PAPS as in yeast. The stimulation of reduction by light and the formation of the reduced C-SS-like compound was thought by Asahi to suggest the coupling of sulphate reduction through this disulphide to the photosynthetic electron transport system.

Tamura (1964) found that crude leaf extracts from several plants reduce sulphite to sulphide with MVH. After fractionation of the extracts several of the fractions showed some NADPH- linked activity whereas the crude extract was not only inactive with NADPH but also inhibited the activity of yeast sulphite reductase. Tamura (1965) reported the purification of sulphite reductase from Allium with a 190 fold increase of specific activity, but the reduction was linked to MVH. Asada, Tamura and Bandurski (1966) purified the MVH-sulphite reductase of spinach about 180 fold and also cleaved it into two non-dialyzable components which failed to catalyze the reduction separately. The reduction of sulphite by MVH was stimulated in the presence of the enzyme by the addition of ADP and ATP and other nucleoside diphosphates and pyrophosphate. In a preliminary communication of this work (1966), Asada and Bandurski proposed, from this stimulation by nucleosides, that sulphite

reductase was a component of the electron transport system (presumably the cytochromes).

Mayer (1967) examined the distribution of MVH-sulphite reductase in extracts of pea roots and shoots, as well as in the chloroplasts isolated from spinach leaves. He concluded that most of the activity was contained in mitochondria from the roots and chloroplasts from the shoots. Since the enzyme was particulate bound and has a high reducing potential he also suggested it to be a part of the electron-transport system. In these reports the failure of phsiological reducing agents to reduce sulphite throws some doubt on the significance of this enzyme, especially as it could be a component of the electron-transport system.

Bruggemann et al. (1962) reported that extracts of spinach leaves catalyzed the formation of cysteine from sulphide and serine. They also reported the enzyme in E.coli as did Pasternak et al. (1965) who found that it was not repressed by growth on cysteine, whereas the O-acetylserine sulphydrase of Kredich and Thomkins (1966) was. This suggests that the cysteine synthase has no physiological significance.

The formation of radioactive cysteine and glutathione from (^{35}S) sulphate was first reported by Steward, Thompson, Miller, Thomas and Hendricks (1951) in lucerne. Biswas and Sen (1959) found that pea plants fed radiosulphate labelled homocystine, methionine, cystine, taurine and glutathione in the leaves after one hour. The first compounds to be labelled in the leaves, after sulphate, were methionine and taurine, followed by cysteine and homocystine.

Pate (1963) detected (^{35}S) sulphate in the sap of field-pea fed (^{35}S) sulphate, until after one hour (^{35}S) methionine appeared of high specific activity. In extracts of the roots after one hour, protein cystine and methionine were labelled along with free methionine. Pate suggested that methionine was of especial significance in the metabolism of sulphur compounds.

Sinha and Cossins, (1963) fed (^{35}S) sulphate to excised hypocotyls of radish seedlings. After 15 min. cysteic acid and

cystine were labelled. After 30 min. taurine was also labelled and methionine as well in illuminated tissue. They found that illuminating the leaves changed the pattern of labelling of the sulphur amino acids without changing the amount of radioactive organic sulphur. Tomato and coleus leaves produced cystine readily but not methionine even after three hours. They also reported that cell-free extracts, with sucrose and magnesium ions present, incorporated (^{35}S) sulphate into the soluble amino acid fraction. This was stimulated by ATP addition.

Passera, Ferrari, and Cultera (1964) found that Chlorella vulgaris which had been fed radiosulphate for nine seconds labelled an unknown compound only. After 90 sec., compounds of the C_3-S type (cystine, cysteine and cysteic acid) were labelled and after 240 sec. C_4-S (homocysteine and homocysteic acid) and C_3-S-CH_3 and C_4-S-CH_3 compounds were labelled. This sequence suggests that, apart from the unknown, sulphate assimilation into methionine is through cysteine and homocysteine.

Ellis (1963) found that washed beetroot slices incorporated (^{35}S) sulphate into cysteine and glutathione after one hour. This incorporation was stimulated by the addition of serine. From this Ellis considered that cysteine was the first formed organic sulphur compound. As with all the evidence from (^{35}S) sulphate feeding so far discussed, this pattern of labelling can equally well be explained by two homocysteine pools, one of which is invovled in the transfer of S to cysteine after entry from sulphate and the other becomes labelled more slowly and contains more homocysteine so that it eventually is detected and the other is not. This argument is especially valid for most of the reports since, except for that of Passera et al., the period of labelling was longer than 15 min. However in spite of these other possibilities it is simplest to presume that cysteine and not homocysteine is the first formed soluble sulphur amino acid.

The existence of free sulphite and sulphide in sulphate assimilation

Although present evidence indicates that sulphite and sulphide are intermediates in the reduction of sulphate, it is doubtful whether they exist as free intermediates in a soluble pool. Torii and Bandurski (1964) found that the product of (^{35}S) sulphate reduction by an enzyme system of yeast was not free sulphite. The specific activity of sulphite released after the reaction by the addition of acid and carrier sulphite rose to a peak at 30 min. and then declined. This indicated a slow release of sulphite. Some radioactive sulphite was still being collected after 150 min. In the absence of carrier sulphite the enzymic products contained a nondialyzable radioactive substance with low electrophore tic mobility at pH 4.5 and this disappeared when carrier sulphite was added.

Dreyfuss and Monty (1962) found that in Salmonella typhimurium the locus involved in sulphite reduction was also involved in PAPS reduction. Wainwright (1962) reported that yeast extracts reducing sulphite could be divided into six fractions, two of which were identical with the protein fractions A and C of PAPS reductase (Wilson, Asahi and Bandurski, 1961).

From this it is most likely that sulphite, and perhaps also sulphide is bound to a high molecular weight substance during sulphate assimilation and in this case the reported labelling of sulphite and sulphide in mung bean by Asahi et al. (1960, 1961) was a result of the displacement of these groups by the carrier sulphite and sulphide added.

The interconversion of cysteine and methionine

In 1947 Lampen, Roepke and Jones reported that mutants of E.coli unable to utilize cysteine grew on cystathionine, homocysteine and methionine (CH_3-S-CH_2-CH_2-CH(NH_2)-COOH). Some grew on all three, others only on homocysteine and methionine, and yet others only on methionine. The metabolic sequence of conversion between cysteine and methionine appeared to be

cysteine	⟶ cystathionine	⟶ homocysteine	⟶ methionine	(26)
3C-SH	3C-S-4C	HS-4C	CH_3-S-4C	

Horowitz (1950) concluded from studies on mutants that Neurospora converted cysteine through cystathionine to homocysteine before forming methionine from the latter.

From extracts of rat liver, Selim and Greenberg (1959) and Matsuo and Greenberg (1958) purified enzymes catalyzing the following reactions and requiring pyridoxal phosphate:

β-replacement (a)

L-serine	+ L-homocysteine	⟶ cystathionine	+ H_2O	(27)	
3C-OH	HS-4C	3C-S-4C			

γ-elimination

L-cystathionine	⟶ L-cysteine	+ α-ketobutyrate	+ NH_3	(28)	
3C-S-4C	3C-SH	$CH_3CH_2COCOO^-$			

(a) The nomenclature is based on the position of the carbon atom on which the electronegative leaving group is situated (Flavin, 1963).

cysteine

α β

HOOCCH(NH_2)CH_2-SH

homocysteine

α β γ

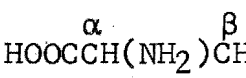
HOOCCH(NH_2)$CH_2$$CH_2$-SH

cystathionine

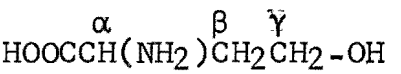
α β γ β α

HOOCCH(NH_2)CH_2-S-$CH_2$$CH_2$CH($NH_2$)COOH

serine



homoserine



The reactions were found to proceed in those directions only thus explaining the essentiality of methionine for animals (Young and Maw, 1958), although cystine exerts a sparing action on the need for methionine.

Flavin and Slaughter (1964) isolated two pyridoxal phosphate requiring enzymes from Neurospora crassa, one of which catalyzed reaction 28 (γ-elimination) and the other reaction 29 (β-elimination)

$$\text{L-cystathionine} \xrightarrow{\beta\text{-elimination}} \text{L-homocysteine} + \text{pyruvate } (CH_3COCO_2H) + \text{ammonia} \qquad (29)$$

Flavin and Segal (1964) purified the γ-elimination enzyme from Neurospora 400 fold. It also catalyzed the degradation of a number of disulphide compounds such as cystine. This and the rapid growth of Neurospora in minimal medium containing sulphate, when such an enzyme would be useless synthetically, caused the authors to suspect the physiological significance of this enzyme. However they noted that the liver enzyme also decomposed lanthionine $HOOCCH(NH_2)CH_2\text{-}S\text{-}CH_2\text{-}CH(NH_2)COOH$, djenkolic acid and cystine as did the Neurospora enzyme, and pointed out that the derivation of cysteine from methionine is essential in mammals for which no other pathway than that involving cystathionine is known.

From E.coli Rowbury (1962) isolated an extract catalyzing the formation of cystathionine from cysteine and homoserine (γ-synthetase). He found a stable intermediate was formed in the extracts and tentatively identified it as O-succinyl homoserine. Flavin, Delavier-Klutchko and Slaughter (1964) reported that extracts of Salmonella catalyzed the formation of a compound chromatographically identical to cystathionine from cysteine and O-succinylhomoserine. In the absence of

cysteine the enzyme degraded O-succinylhomoserine to α-ketobutyrate and both the synthetic and decomposition reactions were absent from the same mutant. This indicated that they were catalyzed by the same enzyme.

Rowbury (1964) found that E.coli and Salmonella mutants requiring cystathionine accumulated O-succinylhomoserine and that methionine feeding prevented accumulation in both organisms. The importance of O-succinylhomoserine in cystathionine synthesis was further supported by Kaplan and Flavin (1965) who identified the product of the reaction of O-succinylhomoserine with cystine in Salmonella extract as cystathionine by several means.

$$\underset{\text{3C-SH}}{\text{cysteine}} + \underset{\text{succinyl-O-4C}}{\text{O-succinylhomoserine}} \xrightarrow{\gamma\text{-replacement}} \underset{\text{3C-S-4C}}{\text{cystathionine}} + \text{succinate} \qquad (30)$$

In E.coli, Roberts et al. (1955) found that (^{35}S) methionine was incorporated only into methionine and homocystine by cells whereas (^{35}S) cystine was incorporated into both cysteine and methionine. This indicated that 'transsulphuration' - that is the transfer of the S atom between cysteine and homocysteine - proceeded only from cysteine to methionine and from the results of Lampen, Roepke and Jones (1947) and Simmonds (1948) the intermediates are cystathionine and homocysteine.

To elucidate the interconversion of cysteine and homocysteine, Delavier-Klutchko and Flavin (1965) investigated enzymatic cleavage and synthesis of cystathionine in bacteria and fungi. For E.coli and Salmonella they found γ-synthetase in dialyzed extracts and the reaction was stimulated by pyridoxal phosphate, and also an enzyme catalyzing β-elimination to give homocysteine and pyruvate. They were unable to detect any formation of cysteine from cystathionine, or formation of cystathionine from homocysteine and serine in bacterial extracts. Thus the enzymes present allowed transsulphuration only from cysteine to homocysteine.

Purified Neurospora and yeast extracts catalyzed both β- and γ-elimination so that cystathionine formed either cysteine and α-ketobutyrate or homocysteine and pyruvate. Further Delavier-Klutchko and Flavin (1965) found a β-synthetase in extracts of both Neurospora and yeast which catalyzed the formation of cystathionine from serine and homocysteine. This enzyme was separate in Neurospora from that which catalyzes γ-elimination since the former was alone present in extracts of mutant me-2 of Neurospora. (Mutants me-1 to me-7 refer to mutants blocked between cysteine and methionine. Each number refers to a type.)

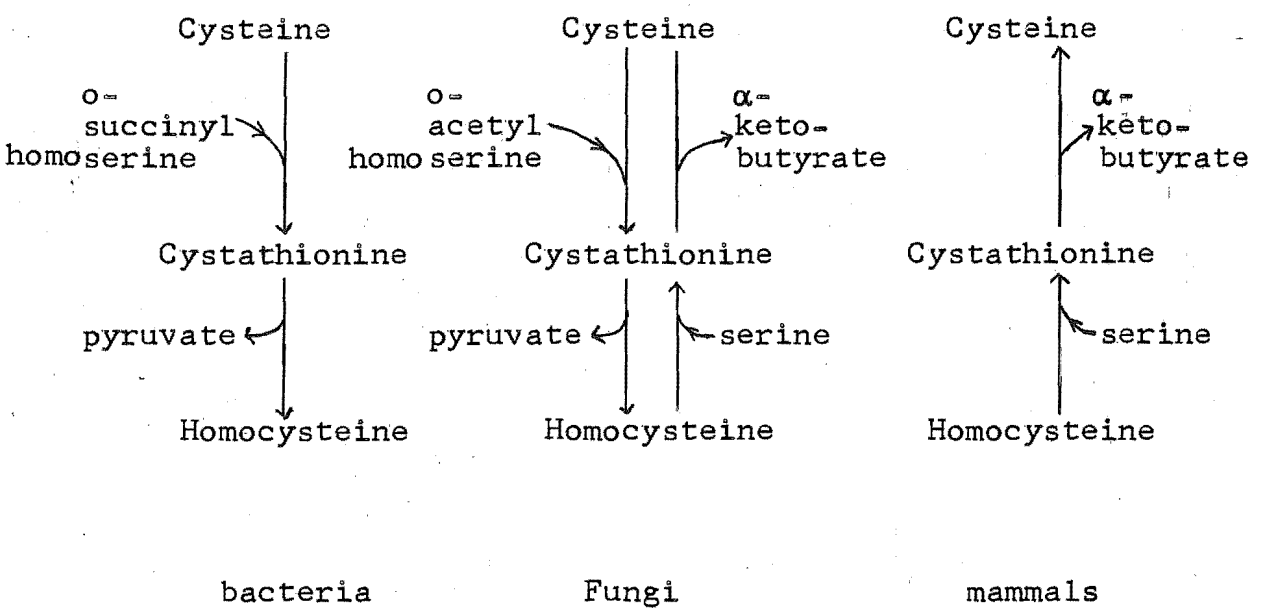
All attempts to establish the synthesis of cystathionine from cysteine and O-succinylhomoserine in yeast and Neurospora extracts failed. Nor did the γ-elimination enzyme of Neurospora catalyze the reverse reaction and so synthesize cystathionine from cysteine and homoserine. Crude extracts of Neurospora and yeast also failed to show cystathionine synthesis from cysteine and homoserine or several other derivatives. Mutants of Neurospora which failed to grow on cysteine but do grow on cystathionine also failed to grow on O-succinylhomoserine. This and the failure of extracts to synthesize cystathionine suggested that O-succinylhomoserine was not an intermediate in cystathionine synthesis, although cystathionineless Salmonella mutants also failed to grow on O-succinylhomoserine.

However Nagai and Flavin (1966) found that mutant me-5 (cystathionineless) of Neurospora grew on O-acetylhomoserine whereas mutants me-3 and me-7 (cystathionineless also) failed to do so. When grown in the presence of methionine and labelled homoserine, me-5 mutants failed to accumulate two compounds which were formed by me-3, me-7 and the wild type, namely O-acetylhomoserine and a larger amount of 4-O-acetyl-2, 4-dihydroxybutyrate. Extracts of me-5 also lacked an enzyme catalyzing an exchange between labelled homoserine and O-acetylhomoserine. These results suggest that O-acetylhomoserine and not O-succinylhomoserine is the appropriate intermediate in cystathionine synthesis by γ-replacement in Neurospora.

$$\text{cysteine} + \text{O-acetylhomoserine} \xrightarrow{\gamma\text{-synthetase}} \text{cystathionine} + \text{acetate} \quad (31)$$

The results of Wiebers and Garner (1967, 1960), from which they concluded that homocysteine was synthesized from homoserine and sulphide are difficult to reconcile with those of Flavin and Slaughter (1964). The latter found that mutant me-2 which was reported by Horowitz (1947) to accumulate cystathionine in large quantities lacked the enzyme catalyzing β-elimination of cystathionine. For these results to be consistent with those of Wiebers and Garner this mutant must also be blocked in the synthesis of homocysteine at the same time as it is blocked in cystathionine cleavage, otherwise it could still grow on sulphate. In fact Wiebers and Garner reported the presence of homocysteine synthetase in a cystathionineless mutant. However, since the intermediates utilized in homocysteine synthesis might be lacking in the mutant studied, this is not conclusive.

To summarize the results of Flavin et al., transsulphuration appears to take place as follows in different organisms.



Methionine is synthesized from homocysteine by either of two enzymes in E.coli (Woods, Foster and Guest, 1966) and in Salmonella (Cauthen, Foster and Woods, 1966). One enzyme was vitamin B_{12} dependent and other was not. In mammalian liver, a methionine synthetase dependent on Vitamin B_{12} has also been partially purified from pig and beef liver by Kerwar, Mangum, Scrimgeour, Brodie and Huennekens (1966). In higher plants Woods, Foster and Guest (1966) reported that crushed leaflets of non nodulated pea seedlings contained only the vitamin B_{12} independent system for which a folic acid derivative was the methyl donor. Magnesium ions were also required for the reaction but S-adenosylmethionine, ATP and reducing system had no effect, whereas the vitamin B_{12} enzyme from E.coli requires S-adenosylmethionine and FADH.

Turner and Shapiro (1961) reported an S-adenosyl-methionine- and S-methylmethionine-homocysteine transmethylase in the seeds of several higher plants. S-methylmethionine ($CH_3S^+(CH_3)CH_2CH_2CH(NH_2)CO_2H$) was more effective as a methyl donor than S-adenosylmethionine.

S-adenosylmethionine + homocysteine $\longrightarrow$ S-adenosylhomocysteine + methionine (32)

S-methylmethionine + homocysteine $\longrightarrow$ methionine + methionine (33)

Shapiro (1966) recorded that S-adenosylmethionine-methyl transferase was present in extracts from rat kidney and liver, and E.coli K-12, and had been isolated from extracts of Aerobacter aerogenes and Saccharomyces cerevisiae. The S-adenosylhomocysteine produced in the first reaction would have to be converted into S-adenosylmethionine again or the reaction would have no physiological significance. However Schlenk (1966) recorded that his group had fed labelled S-adenosyl-L-homocysteine to yeast cells which incorporated the label into S-adenosyl-methionine. Labelled adenosine was incorporated into S-adenosyl-methionine hardly at all so it appeared that S-adenosylhomocysteine was methylated directly.

Because sulphate is a source of sulphur for higher plants, transsulphuration proceeds at least from cysteine to homocysteine, provided that homocysteine synthetase is not important. Giovanelli and Mudd (1966a) examined the cell free extracts of a number of plant tissues and found that the rate of degradation of cystathionine to pyruvate (and so homocysteine) was much faster than that to α-ketobutyrate (and so cysteine). Hence it seemed that cysteine to homocysteine was the only direction possible. Further Dougall and Fulton (1966) reported from isotope competition studies that synthesis of methionine by "Paul's Scarlet" rose tissue culture followed the pattern:

homoserine ⟶ cystathionine ⟶ homocysteine ⟶ methionine (34)

Chisholm and Wetter (1966) found that (2-^{14}C) homoserine was incorporated into sinigrin. The atom labelled in the allyl isothiocyanate moitey of sinigrin by homoserine was the same as that labelled by (2-^{14}C) methionine. Little randomization of the label occurred. This suggests that homoserine is a precursor of methionine. Support for the synthesis of homocysteine from cysteine was provided by Giovanelli and Mudd (1966b) who found that acetone powder extracts of spinach catalyzed the labelling of cystathionine from (^{14}C) cysteine or (^{35}S) cysteine and O-succinylhomoserine or O-acetylhomoserine. The two serine derivatives were equally effective. Although the authors did not show net synthesis of cystathionine, they considered that it was very likely.

Although Giovanelli and Mudd found little evidence for the transformation of homocysteine into cysteine and thus for a possible pathway of methionine transformation into cysteine, Sugii, Nagasaura and Suzuki (1963) found that from (^{35}S) methionine, garlic labelled S-methylcysteine and S-methylcysteine sulphoxide (possibly formed from the former by atmospheric oxidation as well as by enzymic oxidation, Arnold and Thompson (1962)). Since no (^{35}S) cystine was

present Sugii et al. suggested that S-methylcysteine (SMC) was labelled from methionine forming methyl sulphide. Challenger and Charlton (1947) found that Scopulariopsis brevicaule produced methyl sulphide from methionine. Wolf, Black and Downey (1956) found that yeast extracts synthesized SMC from methyl sulphide and serine. However Thompson and Gering (1966) found in radish leaves that the label of (^{35}S) cysteine was incorporated into SMC and that radioactivity in SMC from (^{14}C) serine was 1/10 that of (^{14}C) cysteine. From this they concluded that the yeast system does not operate in plants. Further the number of counts found in SMC sulphoxide from (1-^{14}C) serine was little different from that found from (3-^{14}C) serine and only about 10% of the counts were in the methyl group. Therefore the counts in SMC from serine were not principally due to serine-hydroxymethylase.

The incorporation of the (^{35}S) methionine label appeared to be via cysteine as the addition of cysteine completely eliminated incorporation of (^{35}S) into SMC and the labels from (^{35}S) methionine and (methyl-^{14}C) methionine supplied together were incorporated to different extents, as were the labels from (^{35}S) methionine and (methyl-^{3}H) methionine.

$$\underset{4C\text{-}S\text{-}CH_3}{\text{methionine}} \longrightarrow \underset{3C\text{-}SH}{\text{cysteine}} \longrightarrow \underset{3C\text{-}S\text{-}CH_3}{\text{S-methylcysteine}} \qquad (35)$$

Since the formation of cysteine from methionine does not involve SMC, two other pathways are suggested, both involving homocysteine. Doney and Thompson (1966) found that turnip leaves supplied with (^{35}S) methionine for four hours contained 10-15% of the radioactivity in homocystine, so that the formation of homocystine does not appear to be limiting. Transsulphuration of homocysteine to cysteine is the first pathway but since Giovanelli and Mudd (1966a) found little evidence for transsulphuration the other possibility is the desulphuration of homocystine to give an inorganic sulphur compound. Tishel and Mazelis (1966) found that

purified extracts of cabbage decomposed cystine to form pyruvate and ammonia and that homocystine was also decomposed about ten times more slowly than cystine.

cystine ⟶ cysteine + S^o + pyruvate + ammonia (36)

homocystine ⟶ homocysteine + S^o + pyruvate + ammonia (37)

The products of the reaction apart from ammonia and pyruvate were not established and the sulphydryl content did not increase so it appears, from analogy with Flavin's (1962) reporting that reactions 36 and 37 were catalyzed by cystathionase preparations from Neurospora, that sulphur was formed. However Wetter (1964) found that in horseradish leaves (^{35}S) methionine contributed almost no radioactivity to sulphate and that sulphide and sulphate were incorporated in the same way so that this pathway seems unlikely. Although it is not possible to conclude that transsulphuration from homocysteine to cysteine does take place the failure of Giovanelli and Mudd to find a γ-cleavage enzyme is not conclusive either.

Chapter 2

The choice of techniques

In the investigation of methionine and cysteine synthesis in microorganisms the use of nutritional studies with mutants has been especially valuable. As yet auxotrophic mutants of higher plants are not available (with one or two exceptions) but a nutritional investigation with those sulphur compounds which are stable in solution would at least suggest possible intermediates in sulphate assimilation. Several sulphur compounds can be proposed as intermediates from work with microorganisms and it seemed possible that higher plants might incorporate some of them, in view of reports that methionine is assimilated by non-sterile plants (Formin and Astakova, 1959) and that cystine is incorporated by sterile plants (Tanaka, 1931).

For the unequivocal establishment of a compound's assimilation the plants must be free from microorganisms. Sterile plants have been infrequently cultured but excised roots offered an alternative. These grow in a completely defined medium and a large clone of root material can be established, so removing genetic variability. Some of the excised roots cultured grow quickly and can be established in an experiment with a small inoculum so that the sulphur reserve of seeds can be avoided.

Since grasses compete more strongly with clovers for available sulphur under low sulphur conditions (Walker and Adams, 1958), and since this might be related to an ability to utilize sulphur compounds unavailable to clovers, a monocotyledon was chosen first for the investigation of its nutrition. Of the monocotyledenous roots cultured, none of which were grasses, Petkus rye was the fastest growing so that its establishment in excised root culture was attempted.

Since this was successful, the preliminary investigations were carried out with excised roots of rye, but later when their physiological vigour declined, tomato roots were used as they were available and other monocotyledonous roots cultured were too slow growing.

The use of labelled compounds was then chosen to establish the pathway of their incorporation once assimilation of these compounds had been established. Sulphate has been used several times as a tracer for higher plants but labelling of sulphur compounds has usually taken several minutes (see Introduction). The use of fast growing root material in which transport of sulphate to the shoots did not occur would help overcome this.

Isotope competition studies have indicated the importance of sulphite, sulphide, cystine and homocystine in sulphate assimilation by bacteria and it was intended to use this technique with excised roots. However it was found that of the compounds studied, only sulphate and the end product methionine was assimilated readily so that other compounds would be unlikely to markedly reduce the incorporation of sulphate. Nutritional and tracer studies were therefore concentrated upon as a means of elucidating the major pathways of sulphur assimilation.

Chapter 3

Methods and Materials

3-1

Culture vessels

The rye root and tomato root clones were maintained in either 250 or 100 ml. Pyrex Erlenmeyer flasks respectively, containing 50 ml. of medium.

Culture experiments were carried out in 100 ml. flasks containing either 50 or 25 ml. of medium. For radio-isotope experiments twelve roots were grown in 300 ml. of medium in 500 ml. flasks.

Flasks were plugged with cotton wool bungs covered with two layers of starch free cotton gauze. New plugs were formed and leached of any soluble materials by autoclaving in the appropriate clean flasks containing distilled water. To keep off water during autoclaving and dust during storage, bungs were covered with numbered aluminium beakers. Care during autoclaving prevented bungs being wetted by medium and thereby transferring sulphur compounds from treatment to treatment.

Cleaning of glassware for culture experiments

All glassware used in the preparation of media for these experiments was cleaned first by thorough rinsing with water then by coating the inside with strong sulphuric acid-dichromate mixture. After standing with chromic acid for not less than half an hour, vessels were rinsed thoroughly for at least 5 min. in running tap water and washed twice with single distilled water. Culture flasks, test-tubes and other small items were washed in tap water on the acid resistant plastic apparatus described by Reay and Sheat (1965).

Cleaning of glassware for radio-isotope experiments

The Pyrex glassware used in these experiments was kept separate from other glassware. After use all radio-isotope solutions were disposed of and vessels were rinsed after which

they were soaked in strong chromic acid at least overnight. They were thoroughly washed in running tap water before rinsing twice in distilled water. Later this procedure was modified to first soak the glassware for about half an hour in chromic acid followed after rinsing in tap water by soaking for 24 hr. in 2% 'Decon' 75 (a proprietary preparation with surface active properties). After washing and rinsing in distilled water as above the glassware was dried in an oven.

Cleaning of Thin Layer glass plates

Glass plates used for chromatography or electrophoresis were cleaned first with 'Chemico' followed, after rinsing clean, by soaking for 24 hr. in an alcoholic solution of sodium hydroxide (20 g. caustic soda in 120 ml. distilled water made up to 1 l. with absolute alcohol). Later to avoid the brown precipitate which the alcoholic sodium hydroxide solution tended to leave on the plates, these were soaked in 2% 'Decon' 75 for 24 hr. After either treatment, the plates were washed in hot water, rinsed with distilled water and dried in the air.

3-2

Media used for the culture of rye and tomato roots

In this work the media compiled by several authors as well as modifications to these media have been used at different times. These are designated by numbers, more or less in the order in which they were used, and are referred to either by the names of the authors or by some characteristic of the medium. Thus Charles and Street's medium (Medium I) refers to the nutrient solution used by Charles and Street (1959) for the culture of groundsel roots. The exact composition of the inorganic components of these nutrient solutions is given in the text as parts per million of the element. The vitamin components are similarly given as ppm. but the sugar content is given as a percentage on a w/v basis, that is 2% sucrose is 20 g. of sucrose in 1000 ml. of final medium. The preparation, of the media, the components of the stock solutions and their storage are described in Appendix 1.

(a) Constituents of the media referred to frequently

Vitamin supplement

The vitamin supplement used by White (1943) for tomato roots and by Roberts and Street (1955) for rye roots, was used for all media. The concentration of each vitamin is set out below and details of the stock solutions are to be found in the Appendix 1-1.

Glycine	3 ppm.
Thiamine HCl	0.01ppm.
Pyridoxine	0.01ppm.
Nicotinic acid	0.5 ppm.

Charles and Street's micronutrients

The micronutrient elements of Charles and Street (1959) were prepared in one stock solution. At first the salts as described by them were used (Variant A) but after Experiment 3 had shown the presence of a considerable sulphate impurity in the medium the sulphate salts were replaced by their respective chlorides.

Element	Final concentration (ppm.)	Salt added	
		Variant A	Variant B
Zn	0.64	$ZnSO_2.7H_2O$	$ZnCl_2$
I	0.58	KI	KI
Bo	0.262	H_3BO_3	H_3BO_3
Mn	0.044	$MnCl_2$	$MnCl_2$
Cu	0.005	$CuSO_4$	$CuCl_2$
Mo	0.001	H_2MoO_4	H_2MoO_4

(b) Media

3-3 Media for the growth of rye roots

Medium I Charles and Street's (1959)

Element	Final concentration (ppm.)	Salt added
Ca	48.8	$Ca(NO_3)_2$
K	65.4	KCl, KNO_3
Na	67.8	NaH_2PO_4, Na_2SO_4
Mg	73.0	$MgSO_4$
Cl	32.6	KCl
N	49.7	$KNO_3, Ca(NO_3)_2$
P	4.28	NaH_2PO_4
S	143	$MgSO_4, Na_2SO_4$
Fe	1.0	Fe EDTA[a]

plus 2% glucose, Micronutrients A and the vitamin supplement.

[a] ethylene diamine tetra acetate (EDTA)

Medium II Murashige and Skoog's (1962)

Element	Final concentration (ppm.)	Salt added
Ca	121	$CaCl_2$
K	36.4	KNO_3, KH_2PO_4
Mg	782	$MgSO_4$
N	841	KNO_3, NH_4NO_3
Zn	1.95	$ZnSO_4$
I	0.634	KI
Bo	1.08	H_3BO_3
Mn	5.49	$MnSO_4$
Cu	0.0064	$CuSO_4$
Cl	220	$CaCl_2$
Fe[a]	1.0	Fe EDTA
P	38.8	KH_2PO_4

a Since excess iron was likely to be toxic, the iron concentration of Murashige and Skoog was replaced by that of Charles and Street.

The medium contained in addition 2% glucose, yeast extract and vitamin supplement.

Medium III 'rye clone' medium

Bonner's (1940) inorganic medium with Charles and Street's micronutrients.

Element	Final concentration (ppm.)	Salt added
K	71.2	KCl, KNO_3
Ca	40.1	$Ca(NO_3)_2$
Mg	3.55	$MgSO_4$
N	39.2	$Ca(NO_3)_2$
P	4.55	KH_2PO_4
S	5.53	$MgSO_4$
Cl	31.1	KCl
Fe	0.35	Fetartrate

In addition the medium contained Micronutrients B 2% glucose, vitamin supplement and 60 ppm. yeast extract, unless otherwise stated.

Medium IV 'tryptophane' medium

Modified Bonner's (1940) medium with Charles and Street's micronutrients.

After the 'rye clone' medium was used for the maintenance of the clone, this medium was used for experiments with rye roots until Experiment 2 was completed. After this Experiment the medium contained the same concentrations of macronutrients except for nitrogen, but different salts were added to the medium. The nitrates of calcium and potassium were replaced by their respective chlorides and nitrate was added as the sodium salt. Magnesium sulphate was replaced with magnesium chloride and sodium sulphate. To ensure the availability of iron, ferric tartrate was replaced with Fe-EDTA in which the iron was at the same concentration and EDTA was added in a 1:1

Element	Final concentration (ppm.)	Salt added
K	71.2	KCl, KH_2PO_4
Ca	40.1	$CaCl_2$
Mg	3.55	$MgCl_2$
Na	48.0	$NaNO_3, Na_2SO_4$
N	25.0	$NaNO_3$
P	4.55	KH_2PO_4
S	5.53	Na_2SO_4
Cl	140	$KCl, CaCl_2, MgCl_2$
Fe	0.35	FeEDTA

In addition the medium contained Micronutrients B, 2% glucose, 0.54 ppm. tryptophane and vitamin supplement.

3-4

Media for the growth of tomato roots

Medium V 'tomato clone' medium

Bonner's (1940) medium plus Charles and Street's micronutrients.

Element	Final concentration (ppm.)	Salt added
K	71.2	KCl, KNO_3
Ca	40.1	$Ca(NO_3)_2$
Mg	3.55	$MgSO_4$
N	39.2	$Ca(NO_3)_2$
P	4.55	KH_2PO_4
S	5.53	$MgSO_4$
Cl	31.1	KCl
Fe	0.35	Fetartrate

In addition the medium contained Micronutrients B with 1.5% sucrose and vitamin supplement.

This medium was used for the culture of the tomato clone until Mwauluka (1967) found that increasing the magnesium concentration increased the growth rate of tomato roots.

Medium VI 'low N' medium

Modified Bonner's (1940) medium as for 'tryptophane' medium (Medium IV) with Charles and Street's micronutrients.

This medium was used for culture experiments with tomato roots until the magnesium ion concentration was increased. The replacement of calcium nitrate with sodium nitrate as in Medium IV was adhered to, along with the other modifications, since 'Analar' sodium nitrate contained less sulphate impurity than 'LR' calcium nitrate (see Table 4-3). Also the low nitrate concentration was retained since in Experiment 8 growth of tomato roots was satisfactory and the lower concentration of nitrate added less sulphate as an impurity.

Element	Final concentration (ppm.)	Salt added
K	71.2	KCL, KH_2PO_4
Ca	40.1	$CaCl_2$
Mg	3.55	$MgCl_2$
Na	48.0	$NaNO_3$, Na_2SO_4
N	25.0	$NaNO_3$
P	4.55	KH_2PO_4
S	5.53	Na_2SO_4
Cl	140	KCl, $CaCl_2$, $MgCl_2$
Fe	0.35	Fe EDTA

The medium contained in addition Micronutrients B, 1.5% sucrose and the vitamin supplement.

Medium VII 'high magnesium clone' medium

Bonner's (1940) inorganic medium with magnesium chloride and Charles and Street's micronutrients.

This medium was used for the maintenance of the tomato clone used to inoculate Experiments 11-16 and 19-22. To increase the magnesium concentration, magnesium chloride alone was added and not magnesium sulphate, since Martin and Walker (1966) concluded in their review that plants often take up sulphate above their requirements. The increasing of the sulphate concentration might have increased the sulphate in the tips used to inoculate the experiments.

Element	Concentration (ppm.)	Salt added
K	71.2	KCL, KH_2PO_4
Ca	40.1	$Ca(NO_3)_2$
Mg	71.0	$MgCl_2, MgSO_4$
N	39.2	$Ca(NO_3)_2$
P	4.55	KH_2PO_4
S	5.33	$MgSO_4$
Cl	237	$MgCl_2, KCl$
Fe	0.35	Fetartrate

In addition the medium contained Micronutrients B and 1.5% sucrose and the vitamin supplement.

Medium VIII 'high magnesium' medium

Modified Bonner's (1940) medium with increased magnesium chloride and with Charles and Street's micronutrients.

The same replacement of salts in 'tryptophane' medium (Medium IV) was continued for this medium, since it helped to reduce the sulphate impurity, except for an increase of nitrate concentration. The nitrate concentration was increased to Bonner's level because the increased growth of the roots balanced out any extra sulphate impurity from this increase and the lower concentration may no longer have been optimal.

For convenience of description this medium has no sulphate present and in each experiment the appropriate sulphur source is indicated.

Element	Concentration (ppm.)	Salt added
K	71.2	KCl, KH_2PO_4
Ca	40.1	$CaCl_2$
Mg	71.0	$MgCl_2$
Na	48.0	$NaNO_3$
N	39.2	$NaNO_3$
P	4.55	KH_2PO_4
Cl	367	$KCl, CaCl_2, MgCl_2$
Fe	0.35	Fe EDTA

In addition the medium contained Micronutrients B, 1.5% sucrose and the vitamin supplement.

3-5

Carbohydrate Source

The sugar was added to the medium either as a freshly prepared solution or after ion exchange as described below. The media for the growth of the clonal roots always contained untreated sugar.

Purification of sugar by Ion-exchange

After Experiment 4 showed that a sulphur deficiency in excised rye roots could be brought about by ion-exchanging the glucose solution, this procedure was scaled up to handle larger quantities of sugar at one time.

De Acidite FF 50-100 mesh 8% DVB had proved satisfactory in the earlier Experiment, but it was considered that a useful increase in either the flow rate or the capacity could be achieved with smaller beads. The same anion exchanger was used but in 100-200 mesh. The rate at which a viscous sugar solution would flow through the bed of a fine resin (200-400 mesh) was uncertain and an intermediate size which was readily available was chosen.

The media for each week's experiments required a maximum of 200-300 g. of glucose and a quantity of resin to purify this amount in one operation was assessed. From the manufacturer's stated maximum limits for sulphur impurities, it was calculated that not more than 0.1 milliequivalents of sulphate and sulphite ions would be present in 100 g. of glucose.

The quantity of resin was derived from the following considerations. Samuelson (1953) has summarised the break-through capacity of strongly acid resins for various cations. This is the number of milliequivalents of an ion that can be passed into a column before the ion can be detected in the effluent. To measure the breakthrough capacity, a dilute solution of the ion (less than about 0.1N) is passed into the top of the column and the concentrations of the ion in the effluent measured. The breakthrough capacity depends on the proportions of the column, the volume of resin, the degree

of crosslinking, the rate of flow, the temperature, and the size of the beads, as well as on the nature and concentration of the ion(s) being passed through and the nature of the ion on the resin and the detection sensitivity for the ion in the effluent. The longer the column, the greater the volume of resin, the slower the flow rate, the higher the temperature, the smaller the bead and the greater the affinity of the resin for the ion being passed through, the greater will be the breakthrough capacity. The affinity is described by the selectivity coefficient: K_B^A for the equilibrium,

$$A^a + B_r^b \rightleftharpoons A_r^a + B^b$$

where A and B are the ions involved, 'a' and 'b' are their respective charges and the subscript 'r' denotes the resin phase.

K_B^A is defined as $\dfrac{[A]_r^b \cdot [B]^a}{[B]_r^b \cdot [A]^b}$, Samuelson (1963)

Brackets denote the concentration in the external or resin phases. A K_B^A of greater than one means that the external ion is favoured in the equilibrium. The selectivity coefficient depends upon the concentration of the two ions in the resin as well as the external concentration, so that altering the concentration of the ions passing through a column will change the breakthrough capacity.

Information on breakthrough capacities for anion resins is meagre so that data for cation resins was used. Since the selectivity coefficients describe the affinity of an ion for a resin, a cation with a selectivity coefficient similar to those for bisulphate and bisulphite was chosen to enable the breakthrough capacity for the strong base anion resin to be estimated from the appropriate data for strong acid cation resins. It was assumed that the factors influencing the uptake of cations were similar to those influencing the uptake of anions, as suggested by Samuelson (1963).

For a strong base anion resin, the hydroxyl anion has the lowest affinity (BDH booklet). The free base form cannot be used in this application as it catalyzes the oxidation and inversion of glucose (Whitmore, 1951). Wheaton and Bauman (1951) published some selectivity coefficients for a strong base anion resin. The anion with the next lowest affinity to hydroxide and the most physiologically inert is the chloride ion. Bisulphate has a selectivity coefficient of 4.1 compared with chloride and that of bisulphite is 1.3.

The breakthrough capacity is defined for practical purposes as that capacity when the concentration of ion in the effluent is 0.001 of that in the influent.

The breakthrough capacity for lithium which has the lowest breakthrough capacity of those cations mentioned on a 50 ml. column (H^+) is 3 milliequivalents: the highest breakthrough capacity is about 8 times larger than this. Lithium has a slightly lower affinity for the resin than hydrogen ions (selectivity coefficient 0.8), whereas both bisulphite and bisulphate have somewhat higher affinities for the resin than chloride. As the selectivity coefficients may vary greatly with changing relative concentration of the ions as well as with the concentration, it was decided to use the breakthrough capacity for lithium.

If the concentration of counter ion (H^+) is raised to 3 times the potassium concentration, the selectivity coefficient for potassium falls about one third. Sugars contain considerable quantities of chloride - the counter ion in this case - so the breakthrough capacity of the resin was reduced to 1 milliequivalent. This is about three times that required for 300 g. of glucose, so for a safety factor of 12 times it was decided to use 200 ml. of resin.

The concentration of sulphur anions in the sugar is low - about 0.1 mM at the concentration of glucose passed through the column - so the level of sulphate impurity in the regenerant will affect the breakthrough capacity of the column. 'Analar' hydrochloric acid has the lowest sulphate impurity per

mole of chloride ion so that this was chosen in preference to sodium chloride.

Only a small portion of the total exchange capacity of the resin was used so that five volumes of 10% HCl was considered to be sufficient for regeneration. This quantity is usual for normal working of a resin, (BDH handbook).

After regenerating the resin it was washed with about two and a half litres of double distilled water until the pH rose to 3.8-3.9. That it did not rise to 5.6 - the pH of distilled water - is probably due to the exchange of carbonate ions in the distilled water for chloride ions on the resin. This exchange was ignored as carbonate has less affinity for the resin than does chloride, (Wheaton and Bauman, 1951).

The resin was supported on a sintered glass plug in a Pyrex tube. The dimensions of the resin column were 52 x 2.2 cm. Regeneration and washing of the resin were carried out in the upward direction, whereas ion-exchanging of the sugar solution was in the downward direction. All solutions flowed through at less than 1.5 ml./min. Before filling the column with the resin (DeAcidite FF, SRA 71) the latter was purified by three regeneration cycles as described in the BDH handbook. A plug of glass wool was placed on top of the resin to keep it in place.

The sugar solution was run into the top of the column and the effluent run to waste until the sugar solution first appeared in the glass tubing below the column. After this the effluent was collected. When the sugar solution had passed into the column it was washed through with 250 ml. of distilled water. In doing this, it was assumed that relatively little sugar would be held in the column and so the final sugar concentration in the effluent was known. Since the growth of tomato roots and rye roots in media containing ion exchanged sugar was similar to that in media with untreated sugar, this assumption was justified.

3-6

The preparation of solutions of the various sulphur sources

Solutions of the sulphur amino acids were freshly prepared before filter sterilization as described in Appendix 1-5. The colloidal solution of elemental sulphur was prepared after Miller, McCallan and Weed (1953) as described in Appendix 1-5.

Sterilization of media and glassware

Organic sulphur compounds were sterilized by filtration through a Millipore HA type membrane filter and added to the bulk of the medium which had been already autoclaved as described more fully in Appendix 1-4. Media were autoclaved at 15 lbs for 10 min. The Millipore filter was sterilized by autoclaving as described in the manufacturers handbook. All glassware except Petri dishes for the subculturing of the clones were sterilized in the autoclave as for media. The latter were sterilized at 160°C. for 4 hrs.

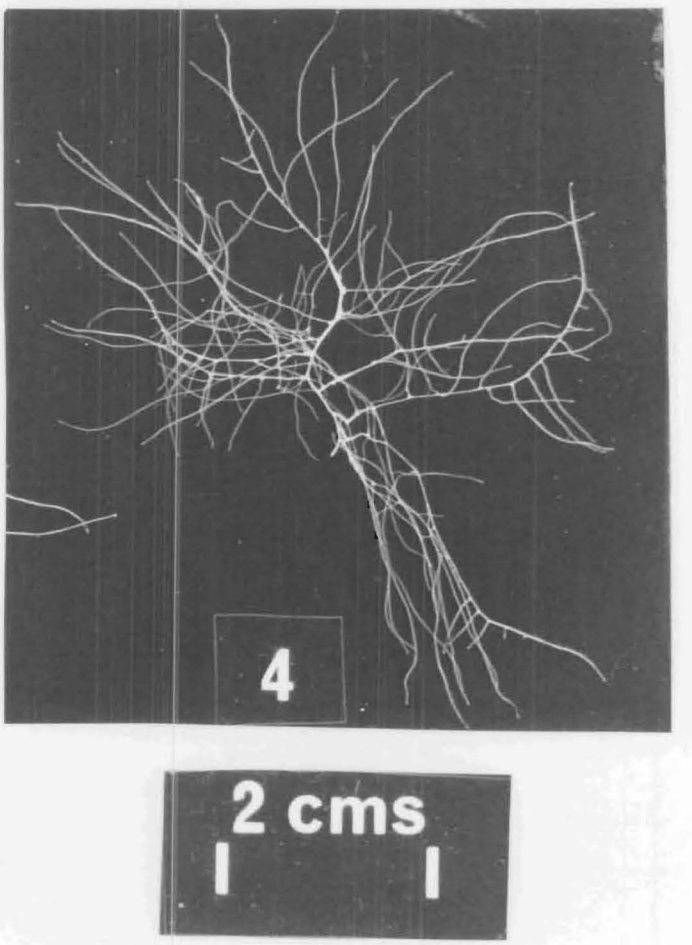
Adjustment of pH

For media whose pH was adjusted after autoclaving, the bulk of the medium was autoclaved and its pH adjusted with sterile 0.1 N sodium hydroxide or hydrochloric acid in the sterile transfer room. After the addition of a little acid or alkali to the medium, the pH of a small portion was measured with a pH meter in the sterile room and then this portion was thrown out. The process was continued until the appropriate pH $\pm$ 0.1 of a unit was reached and the medium was then dispensed. For media whose pH was adjusted before autoclaving with 0.1 N sodium hydroxide, the value quoted for each experiment was an average of the values for treatments, but which were always within 0.1 of a pH unit of the average.

Removal of sulphur from rubber

The sulphur impurity present in rubber was removed from the latex rubber tubing and rubber corks which were used in the preparation of media. These were boiled in 1 N sodium hydroxide for half an hour and rinsed in distilled water five times. Since sulphate diffused into rubber tubing used to dispense

FIGURE 3—7



RYE ROOT TIP WITH MANY SECONDARY LATERALS FROM EXPERIMENT 2.

media, a fresh piece of latex rubber was used for each new solution.

Subculturing of the root clones and inoculation of experiments

The tomato clone was subcultured as described by Sheat (1956). The rye root clone was established and subcultured as described in Methods and Materials (3-7) after Roberts and Street (1955). Tips were removed from sectors after the appropriate growth period of 7 or 14 days and inoculated into the treatments at random. Unless otherwise stated the number of replicates in a treatment was ten.

The growth period

The roots were incubated at 26°C. Clonal sectors of rye roots or rye roots grown in experiments were subcultured or harvested after 14 days. Clonal tomato roots, roots grown during culture experiments and those grown for radio-isotope experiments were harvested or subcultured after 7 days.

3-7

Measurement of growth

After the appropriate periods of growth, measurements were made of the length of roots' main axes to the nearest mm., laterals were counted and the total lengths of primary laterals estimated to the nearest cm. by comparison with a mm. scale set alongside the root.

In Experiment 2 the roots formed many secondary laterals which were not measured when only the lengths of the main axis and primary laterals are determined. Since the secondary laterals contributed considerably to growth and measurement by length was too tedious, the fresh weight of the roots was used as a measure of growth in the second passage. This was continued for rye and tomato roots since the sulphur content of higher plants is roughly proportional to the dry weight (Gilbert, 1951), and the latter was a relatively constant fraction of the fresh weight. For this reason, the dry weight of roots was determined as well, but the fresh weight gain was used as the main parameter of growth because each

individual root could be weighed and from this an estimate of the variability of root growth obtained.

Measurements of fresh weight were carried out by rinsing roots once in distilled water and pressing between two pieces of fresh blotting paper before weighing to the nearest 0.1 mg.

The fresh weight gain was found by subtracting the average weight of a tip (0.85 mg. for a tomato root and 0.7 mg. for a rye root) from the fresh weight unless the tip had been subcultured for a second passage. When the roots were harvested, those which had grown very poorly were not measured.

The procedure for this determination was tested by using a large clone root of rye so that any change of weight would be more easily measured. The root was rinsed, blotted, weighed and then placed back in the distilled water. After 5 min. the root was again weighed in the same way. While on the balance, it was seen to loose weight, presumably by evaporation. It was also found that the amount of pressure exerted on the blotting paper altered the loss of weight and that some of the water lost from the roots was not regained on soaking. The greater the pressure the more weight the root lost, compared with the previous weighing. As too little pressure tended to leave drops of water on the roots, it was decided to accept a small loss of water as a result of this pressure, provided that it appeared reproducible for weighings of the same root.

In the Table are shown the successive weights found for a single large clone root of Petkus rye.

Weight (mg.)	
50.4	47.4
50.2	46.4
49.0	45.8
48.8	46.2
48.4	45.5

Table 3-7i

Vial number	Weight (mg.) for each successive weighing			
	Order of weighing			
	1	2	3	4
1	6.2	5.1	5.7	5.6
2	6.8	7.0	7.6	7.4
3	8.3	8.5	9.2	8.8
4	4.4	4.3	4.6	4.5
5	14.7	14.7	15.1	15.2
6	8.1	8.2	8.5	8.3
7	7.8	8.1	8.0	8.2

The weights of 8 to 10 rye roots in a vial dried at 90°C. for 24 hr. and weighed after four successive dryings and weighings.

Table 3-7ii

Vial number	Weight (mg.) Order of weighing	
	1	2
31	9.21	9.26
32	10.65	10.67
33	8.98	8.97
34	10.25	10.27
35	10.87	10.80
36	9.43	9.85
37	8.54	8.56
38	6.34	6.39

The weights of 8 to 9 tomato roots in a vial dried at 90°C. for 24 hr. and weighed. The drying and weighing was repeated.

It can be seen that the differences between these are fairly similar and are a small proportion of the total weight. The loss of weight from both pressure and drying should be related to the total weight as the cross sectional area of the roots is relatively constant. So it is expected that the loss of weight will be less for the smaller roots. It was found that with practise the loss of weight caused by drying in the air could be reduced to less than 0.5 mg. for a 30 mg. root.

It was concluded from this that this method of weighing gave reproducible results well within the variation of the fresh weight among roots.

The dry weight of all roots was measured in tared, chrome clean and dry four dram vials capped with plastic slip-over lids. As the caps were not heat stable, they were stored in the dessicator used to cool the vials. Vials and caps were numbered.

All the roots of one treatment were placed in one vial as they did not weigh enough individually to provide an reliable estimate. The roots were dried in the oven at 90°C. for at least 24 hr., cooled over silica gel, and the vials recapped. The vials were weighed again and the difference in weight calculated. To estimate the increase of weight of roots with tips, the average dry weight of 20 tips was subtracted from the average weight for each treatment. The average weight for a tip was found to be 0.1 mg. for rye and 0.07 mg. for tomato.

To choose a standard time for the drying of roots, a number were dried for 24 hr., weighed, dried for a further 24 hr. and reweighed. This was repeated twice more. Table 3-7i shows seven lots of roots, each lot containing between eight and ten roots. The weight of the roots did not decrease overall after the first weighing but the variability between the different weighings of the same vial were quite large and the dry weight as a per cent of the fresh weight in the Appendix Tables is expressed only to the nearest one per cent.

Table 3-7ii shows that the weight of tomato roots did not decrease either after 24 hr. drying. With one exception the

results of the second weighing agree within 0.1 mg., so the dry weight as a per cent of the fresh weight is expressed to two figures.

Determination of the significance of the results

The mean and the standard error of the mean were calculated as described in Appendix 2. In the Figures for the experiments the means are plotted along with their standard errors. The vertical bar through the mean is twice the standard error of the mean. The significance of a difference between two means was determined, if intended in the design of the experiment, with the 't' test, calculated as described in Appendix 2. A difference was regarded as significant if the probability of its occurence by chance was 0.05 or less. Since a particular comparison was rarely intended when the experiment was carried out, the following criterion was used as a guide: no difference between two means was regarded as likely to be significant unless greater than the sum of the standard errors of the two means compared.

3-8

Sterilization of seeds

Seeds of Petkus rye for the establishment of a clone, from the Linton Pure Seed Co., Cambridge, England, were first wet by evacuating in distilled water with a drop of detergent for 5 min. and then shaken in 0.1% mercuric chloride at atmospheric pressure for 20 min. The seeds were washed five times by decantation in sterile distilled water.

Although in most cases the germinated seeds appeared to be free from infection, nearly all the tips inoculated into media became infected. Most of these infections were from yeasts although a few were from hyphae growing out of the roots. As seeds of barley, which were difficult to sterilize for yeasts, contained yeast spores within the seed coat (Perera, 1966), it was likely that the same would apply to rye. Crocker (1948) showed that bacteria and fungi are more susceptible to chlorine than the stems and soaked seeds of

higher plants, so an additional sterilization with hypochlorite after the seeds had germinated was included.

Petkus rye seeds were sterilized as before and germinated 48 hr. on sterile filter paper with 10 ml. of distilled water. Immediately before excising the root tips, the seedlings were further sterilized with 2 to 3 ml. of 10% 'Janola' (a commercial sodium hypochlorite containing 3.5% of free chlorine) by adding this to the Petri dish and rocking the latter to mix the Janola with the water. At this time the yeasts would be more active and thus more sensitive. The seed coat had split and absorbed water and this would allow a better penetration of sterilant. The fact that seeds had been germinated on paper out of the water reduced the possibility of yeasts washing around during germination. After this nearly all the tips inoculated into media were free from infection.

Some of these tips became twisted after inoculation into media. As this had not been noted in rye before, it was considered likely that too much Janola was added. The quantity of 10% Janola was reduced to 0.5 ml. on future occasions.

3-9

The establishment of a clone of Petkus rye roots

Roberts and Street (1955) found that growth of excised roots of Petkus rye was optimum in Street's vitamins and inorganic medium which contained yeast extract or tryptophane, and 2% glucose; 4% sucrose was equivalent to 2% glucose as a carbon source.

Their strain of Petkus rye II was not available and Petkus rye 'normal straw' was used in this work. To see whether the same medium was also optimum for the growth of excised roots of this variety some changes in the more important constituents of the medium were compared.

Of the variations that Roberts and Streets (1955) studied the type and quantity of sugar, and the quantity of yeast extract seemed most important. Petkus rye II had grown well with four per cent sucrose or two per cent glucose, so these

Table 3-9

Charles and Street's medium with 25 ppm YE	ITL (mm.)	LN	LL (cm.)	No. of roots harvested
2% Sucrose	9±2.7	2.5±0.75	13±6.3	10
4% Sucrose	12±3.3	2.7±0.62	17±6.3	10
2% Glucose	38±7.9	3.2±0.89	27±9.1	9
250 ppm YE				
2% Sucrose	4±1.4	0.2±0.2	0.1±0.1	10
4% Sucrose	14±1.8	0.2±0.2	0.5±0.5	9
2% Glucose	85±20	17±5.1	68±20	5

The growth during 14 days, of Petkus rye root tips, derived from seeds, in media containing sucrose or glucose at two yeast extract concentrations.

ITL is Increase of total length; LN is lateral number; LL is Lateral length or length of all the laterals.

concentrations of both sugars were included. Two per cent sucrose was tried as well, since tomato roots grew well with this concentration. Yeast extract was added at 25 ppm, which was similar to the 30 ppm used by Roberts and Street, and at 250 ppm, with which Ferguson (1963) found good growth of excised wheat roots.

Instead of Roberts and Street's inorganic medium Charles and Street's medium was used, since it had a lower manganese concentration. The concentration of this element used by Roberts and Street had subsequently been reported by Hannay, Fletcher and Street (1959) to be slightly toxic to tomato roots, although these roots still grew well in media containing the higher concentration.

Experimental details

To Charles and Street's (1959) medium was added quantities of sucrose or glucose, and yeast extract to give the concentration shown below. The initial pH values of the treatments fluctuated quite widely after autoclaving and are set out below. After two weeks the roots were measured through the bottom of the flask.

Supplements to the Medium	Initial pH	No. of replicates
25 ppm yeast extract		
2% sucrose	4.2	10
4% sucrose	4.2	10
2% glucose	4.1	10
250 ppm yeast extract		
2% sucrose	5.1	10
4% sucrose	5.1	10
2% glucose	4.6	5

Results and Discussion

Table 3-9 shows that, after 14 days growth of the roots, two per cent glucose was the most satisfactory sugar source and 250 ppm yeast extract gave better growth of roots with glucose than did 25 ppm yeast extract.

Sectors with three to seven primary laterals were cut from the 14 day old roots in the two per cent glucose media with both concentrations of yeast extract and subcultured into media with the same concentration of yeast extract as described by Roberts and Street.

After 14 days growth few primary laterals were formed on the roots with 25 ppm yeast extract although many were formed on those with 250 ppm of this supplement. The roots with 250 ppm, with many laterals, had the apices of the laterals forming close behind the main apex so that it appeared that the tip might be growing more slowly at the end of the passage. As Roberts and Street chose for the maintenance of the clone a concentration of yeast extract which was well below the optimum; the maintence of this high level of yeast may have inhibited growth over several passages. The yeast extract concentration was therefore reduced to 100 ppm, for the next passage. This concentration was maintained until an experiment on the effect of the concentration of yeast extract on the growth of tips had been completed. (Experiment 1)

When these sectors were subcultured, those which had grown well were selected and of these only those derived from one root were subsequently used. Sectors were about 10-15 mm. long with 3 to 6 primary laterals. Tips from sector laterals were also subcultured and after 7 days roots were divided into sectors. These sectors were subcultured every 14 days, as were the others so that tips for inoculation of experimental media were available each week.

Charles and Street's inorganic medium was used to culture the clone of Petkus rye until after Experiment 1. They were then cultured in a modified Bonner's medium ('rye clone' medium) until the clone was discarded.

3-10

The killing, extraction of tomato roots

The methanol - chloroform - water extractant of Bieleski and Young (1963) was modified to include phosphate buffer at the concentration used by Ellis (1966), for the reaction of thiols with N-ethylmaleimide; 300 ml. of 'Analar' methanol, 125 ml. of 'Pronalys' chloroform and 75 ml. of 1.25 mM phosphate buffer, pH 7.4, were mixed to make 500 ml. of extractant (MCP).

At first, 0.2-0.3 g. of root tissue was extracted in 6 ml. of MCP but the addition of BDH universal indicator showed that the pH fell to six. Although the reaction of thiol compounds with N- ethyl maleimide (NEM), and thus iodoacetamide, was reported by Gregory (1955), to be complete at this pH within 3-4 min. the volume was increased to 10 ml. for Experiment 22, since the pH then fell to 7.0.

To protect thiol groups, iodoacetamide (20 mg.) was dissolved in the MCP in a 50 ml. beaker shortly before adding the 200-300 mg. of root tissue. Ellis (1966) used 50 mg. of N- ethyl maleimide (NEM) to protect thiol groups when extracting 720 mg. of beetroot slices and as he considered this was an excess, an equivalent quantity of iodoacetamide was substituted.

After allowing the roots to stand in the MCP extractant at room temperature for about half an hour to ensure complete reaction of the thiols with iodoacetamide, they were stored in the extractant at less than -15°C. until the roots were homogenized.

The roots were removed from the extractant and transferred to a 'Kontes' glass homogeniser. Fresh MCP (1.5 ml.) was added and the tissue ground at a low speed until a fine slurry was formed. The slurry was transferred to a 15 ml. tapered centrifuge tube with a 'Transpet' and the homogeniser rinsed with 2-3 ml. of 80% ethanol. The washings were also transferred to the centrifuge tube. The slurry was centrifuged at 3200 g. for 12 min. The MCP in which the roots

had been killed and extracted, was transferred to a round bottom flask. To this was added the supernatant from the centrifuge tube. The residue was resuspended in 2 ml. of 80% ethanol with a glass rod. After the glass rod had been rinsed with 80% ethanol, the volume of the slurry was made up to about 10 ml. and mixed by rocking the tube. The residue was again centrifuged down.

The residue was extracted once again with 80% ethanol and then with double distilled water, as described in the preceding paragraph. After the final centriguation, the residue was stored in the centrifugation tube at less than -15°C. until digested with pronase.

All the supernatants were bulked in a round bottom flask and evaporated at less than 35°C. under reduced pressure. The chloroform - methanol soluble lipids were not removed by splitting the extract into two phases in case any of the sulphur compounds were soluble in this solution. After the extract had been evaporated to dryness, it was taken up in about 0.5 ml. of ten per cent isopropanol by running this solution around the inside of the flask. It was then allowed to drain to the bottom of the flask and frozen to coagulate insoluble material. This was then centrifuged down at 3200 g. for 15 min. and the supernatant applied to the cation exchange column or stored at less than -15°C.

3-11
Ion exchange of Extracts

To purify extracts of rye roots from Experiment 17 and tomato roots from Experiment 18 the ion exchange procedure of Thompson, Morris and Gering (1959) was modified to use one cation column as described by Plaisted (1958). Since strong acid cation columns do not retain sulphonic acids at neutral pH such as cysteic acid and glutathione sulphonic acid, the extract was then passed through an anion column to take up these acids as described for organic acids by Nordman and Nordman (in Smith, Vol I, p. 282).

The following procedure was adopted for these extracts and later modified. Two columns containing ten millilitres (0.9 x 15 cm) of Zeocarb 225, 52-100 mesh, 8% DVB (SCR 14) were prepared. Each had a 100 ml. flask fused to the top and a siphon of medical grade PVC tubing to prevent the resin drying out. The top of the resin was covered with four discs of Whatman No. 4 filter paper. The resins were regenerated with 75 ml. of ten per cent 'Analar' hydrochloric acid and washed with 150 ml. of double distilled water.

Two anion columns, 3 x 0.5 cm., were prepared in glass tubes 12 cm. long, constricted at the bottom and plugged with glass wool, and fused at the top to a 2.6 x 7 cm. test tube. This volume of resin (0.6 ml.) was used to exchange the extract from 0.3 g. fresh weight of roots as Ranson (in Paech and Tracey, p. 542 Vol. II, 1955) stated that one millilitre of resin takes up organic acids quantitatively from one gram of plant tissue. Twice the estimated quantity of resin was used as the column was so small. The resin was regenerated with 10 ml. of 2N sodium formate and washed with 10 ml. of double distilled water.

The extracts were added to the cation columns and washed through into the anion column with three portions of 10 ml. of double distilled water. The amino acids were eluted by adding 2N ammonia until the effluent became basic (20 ml.) and then washing with 5 ml. of water. All solutions were passed through at less than a millilitre per minute. The more basic amino acids were eluted with 35 ml. of 4N ammonia, followed by 15 ml. of double distilled water, and the two amino acid fractions were bulked before evaporation, (A.A. fraction).

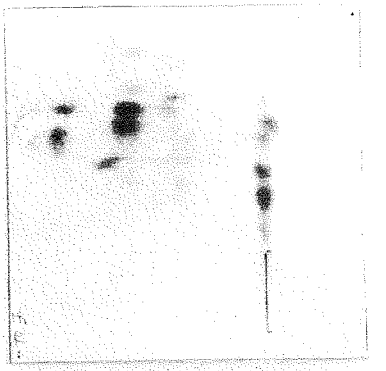
After the washings from the cation column had passed through, the anion column was washed with 3ml. of double distilled water. Substances which resemble weak acids were eluted with 10 ml. of 12 N formic acid (HF.), rinsed through with 1 ml. of double distilled water, (Formic acid fraction). Strong anions such as sulphate were eluted with 9 ml. of 2 N sodium formate (NaF.) followed by 2 ml. of double distilled

FIGURE 3-11 i—iv

(i)EXTRACT OF RYE ROOTS

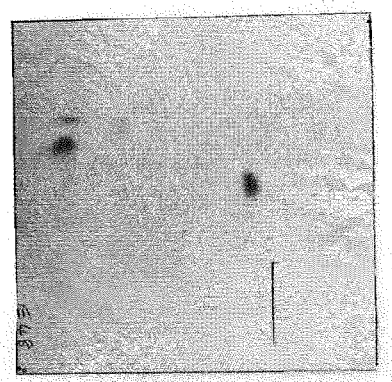
+ve ⟵ pH 5.3 ⟶ -ve

PW



(ii)AMINO ACID FRACTION OF THE EXTRACT SHOWN IN (i).

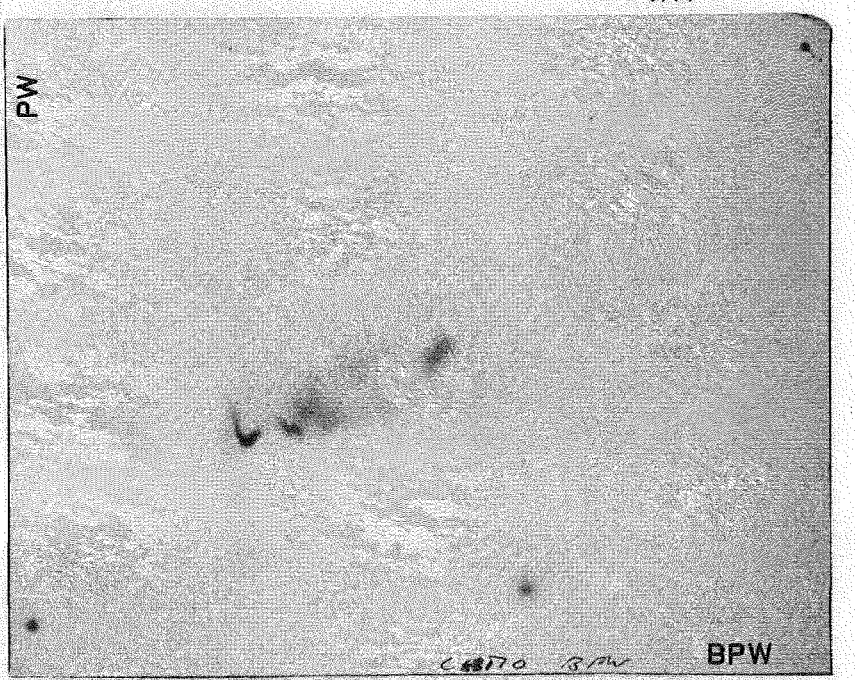
(i) & (ii) EXPERIMENT 17



(iii)

(i),(ii) & (iii) AUTORADIOGRAMS OF THIN LAYER SEPARATIONS.

(iv)



(iii) & (iv): AMINO ACID FRACTION FROM THE TOMATO ROOTS EXTRACTED WITH MCF IN EXPERIMENT 18.

(iv) AUTORADIOGRAM OF A PAPER CHROMATOGRAM. FIRST DIRECTION: BPW.

water, (NaF fraction). To remove the sodium formate, the solution was passed through a cation resin (Zeocarb 225, 25-50 mesh, 8% DVB) in the hydrogen form. The resulting formic acid was washed out with four 10 ml. portions of double distilled water and removed from the eluate with the rotary evaporator.

The sufficiency of 9 ml. of sodium formate for the elution of strong anions, chiefly sulphate, was established from the absence of radioactivity after 7 ml. for one extract.

After each fraction was evaporated, 0.25 ml. of 10% isopropanol was added to the flask and transferred to a vial with a 'Transpet'. The latter was stored at less than -15°C.

Conclusions

From Figure 3-11i which shows the extract of rye roots before ion exchange and Figure 3-11ii which shows the same extract afterwards, it was concluded that sulphur compounds in the amino acid fraction were satisfactorily separated by electrophoresis followed with chromatography. Tomato root extract was also separated after ion exchange as shown in Figure 3-11iii. Many of the compounds shown in Figure 4-18ii are not visible in Figure 3-11iii after ion exchange, because the extracts had been stored for six months before purification.

Two dimensional chromatography was however still unsatisfactory as the compounds streak to some extent and were often distorted (Figure 3-11iv). It appeared that the other amino acids were present in such large quantities that they influenced the mobility of the sulphur compounds.

Modified ion exchange procedure

The former procedure was later modified to purify acetamido- derivatives. Amides are readily hydrolyzed by acid or alkaline conditions, or by ammonia, so the procedure of Thompson et al. (1959) was followed in which they reported quantitative recovery of the amides glutamine and asparagine.

To reduce hydrolysis of amides and oxidation of sulphur compounds, ion exchange was carried out at less than 5°C. and

the columns were always washed with boiled double distilled water to also prevent oxidation (boiled double distilled water is referred to as water). At the same time, the neutral amino acid fraction containing the acetamido-derivatives, after it had been washed through with 10 ml. of water, was evaporated separately from the basic amino acid fraction so that the ammonia was removed within a shorter time after elution of the fraction from the column.

After ion exchange the eluted fractions were evaporated at less than 35°C. under reduced pressure. The 2N ammonia eluate containing the neutral amino acids was evaporated within 3 hr. of elution from the column. The basic cation fraction and the 12N formic acid eluate was evaporated within 8 hr. whereas the HCl and sodium formate eluates were evaporated within 24 hr. as it was considered that no acetamido-derivatives would be present in these. After the eluate had been taken to dryness, the residue was dissolved in about 0.5 ml. of 10% isopropanol and transferred to a 4 dram vial. The solution was then freeze-dried to remove residual ammonia or acid in a vacuum dessicator connected through a dry ice-ethanol trap to a rotary vacuum pump. Until they were freeze-dried the extracts were stored at less than -15°C. for not more than three days, although in most cases the cation column fractions were freeze-dried within six hours and the anion column fractions within 24 hr. It was possible that the amide group could be hydrolyzed as this temperature may not be low enough to freeze all the water in the extract. When ice separates on freezing, the concentration of salts in the solution rises and so the freezing point falls.

Sometimes the strong anion fraction still contained sodium formate which the cation resin had not converted in to formic acid. To overcome this difficulty, the anion resins were later eluted with 10% hydrochloric acid in place of 2N sodium formate. The hydrochloric acid was readily volatile in the 'Buchi' at less than 35°C. but its use had the disadvantage that the anions were present in a

strongly acid solution. Although sulphuric acid is such a strong acid that only a small fraction exists as the undissociated form, freeze drying the HCL fraction of Experiment 22 (^{35}S treatments over sodium hydroxide flakes caused the sodium hydroxide to become radioactive.

After this had been noticed, ammonium hydroxide was added to the flask on the 'Buchi' when it had just reached dryness. A small amount of ammonium chloride was formed at the same time, but this did not appear to interfere in the electrophoresis of the residue. Although the evaporation was carried out under reduced pressure, it was not likely that sulphuric acid would have been significantly volatite at this temperature and pressure.

3-12

Digestion of Residual root material with Pronase

The procedure of Nomoto, Narashi, and Murakami (1960) was used as a guide to develop the following procedure for the hydrolysis of proteins.

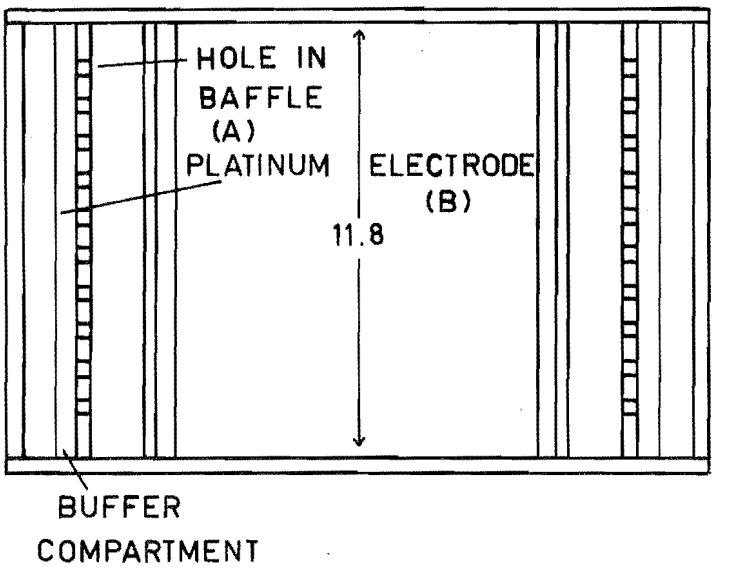
The residue of the root material (corresponding to 0.2 to 0.3 g. of the fresh weight) was digested with 5 mg. of Pronase (Grade B, Calbiochem) in 5 ml. of phosphate buffer, pH 7.4, containing 50 µg. chloramphenicol/ml. to inhibit the growth of bacteria. The residue was shaken with the enzyme preparation in a sealed glass tube for 72 hr. at 37°C., then frozen to coagulate insoluble material, and centrifuged at 2000 g. for 30 min. The supernatant was freeze dried and taken up in 0.2 ml. of ten per cent isopropanol, which was stored at less than -15°C.

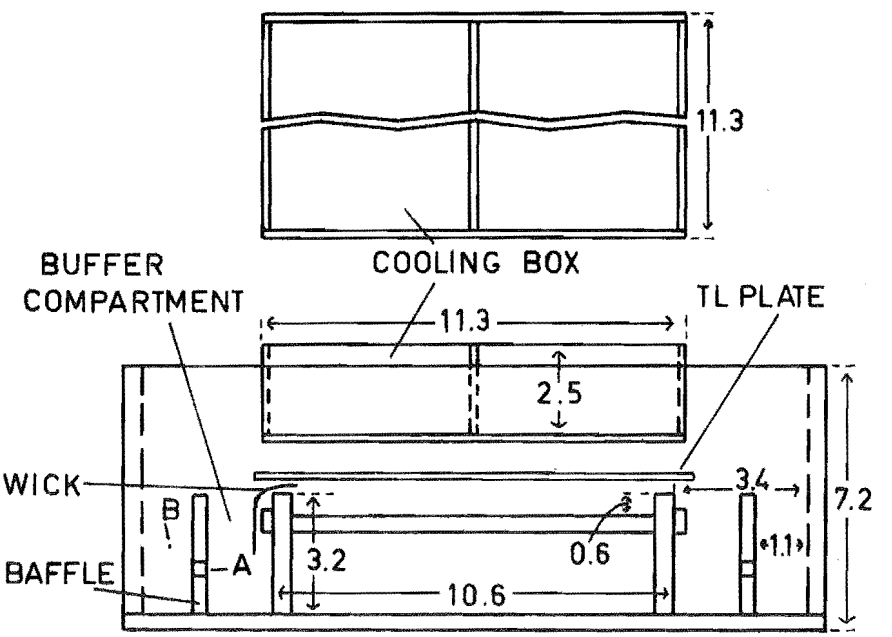
3-13

Preparation of Thin layer plates

Silica gel H (Merck), cellulose powder (Macherey Nagel MN300), and distilled water (4:10:80 by weight, Bieleski and Turner, 1966) were homogenized and spread on to either 11.5 x 11.5 cm. plates with a 'Perspex' spreader or on to 20 x 20 cm. plates with a 'Shandon Unoplan', as detailed more fully

FIGURE 3-14i





PERSPEX APPARATUS FOR ELECTROPHORESIS WITH 11.5 x 11.5 cm. TLPLATES. (All dimensions are in cm.)

The Thin layer plates (TL plates) were left to air-dry and then thoroughly dried with a fan at room temperature. They were used without further preparation until shortly after 20 x 20 cm. plates were first used.

After chromatography in PW, a thin streak of radioactivity was visible at the front edge of the 1% acetic acid used for eluting up the bands, and this became denser after the band length was increased on 20 x 20 cm. plates; a yellow colouration at the same front also became more noticeable and this was associated with the presence of the radioactivity, as it was not soluble in the PW solvent.

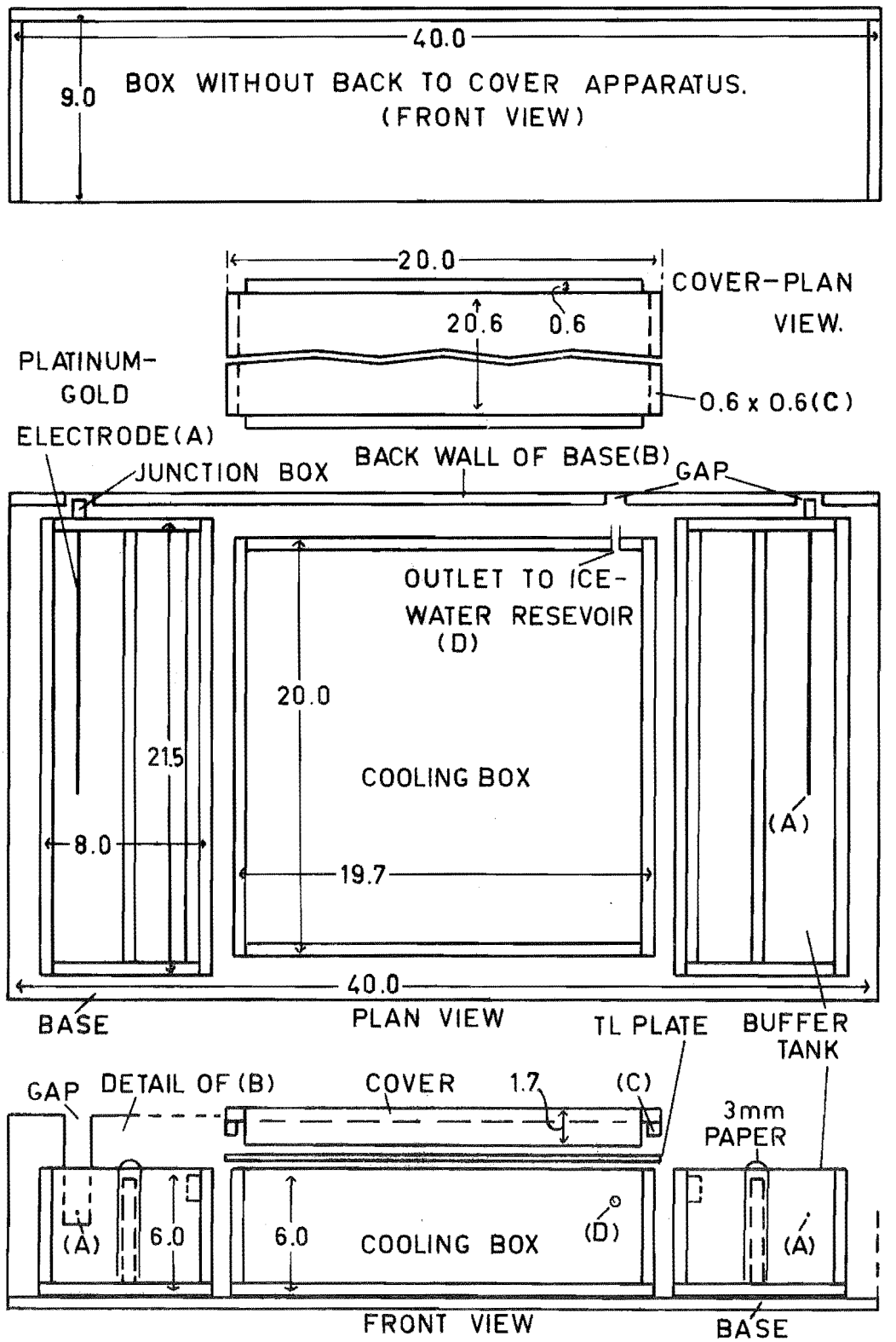
To remove this yellow impurity, plates were dipped in 1% acetic acid followed by two soakings in distilled water, one minute for each, and air dried. This greatly reduced the intensity of the radioactive band and the streaking of spots during chromatography. This rinsing procedure was adopted prior to the separations on 20 x 20 cm. plates shown in the Figures.

3-14
Electrophoresis

The method of Bieleski and Turner (1966) was slightly modified for the electrophoresis of sulphur amino acids. In their method the plates were cooled with a hydrocarbon, "Varsol" (a light petroleum fraction), but this coolant was unsatisfactory for electrophoresis at pH 5.3 as the buffer (pyridine and acetic acid) was appreciably soluble in it, and some of the sulphur compounds might have dissolved in it so making it radioactive. However a satisfactory separation of some amino acids on 11.5 x 11.5 cm. plates was achieved using the technique of Bieleski and Turner with a bag of ice-water slurry to cool the plate. An apparatus for electrophoresis of these plates was made from Perspex and is shown in Figure 3-14i.

To cool the plates more evenly, the plastic bag was replaced with a box, containing the ice-water slurry, made of thin perspex. Evaporation of buffer from the plate was

FIGURE 3-14 ii



APPARATUS FOR ELECTROPHORESIS WITH 20 x 20 cm. TL PLATES
(All dimensions are in cm.)

reduced with a false floor 6 mm. below the surface of the plate.

Electrophoresis of 20 x 20 cm. plates was carried out in the slightly modified apparatus, of Katz and Lewis (1966) according to the method of Bieleski and Turner. The plates were cooled directly with ice-water slurry contained in a Perspex box, Figure 3-14ii, which was made slightly larger than the original, to cool the plate more effectively. A disadvantage of this size was that the cooling water moved on to a side edge of the plate when a potential was applied. Eventually this flooding was prevented by overlapping the plate on that side and drying the edge. In place of the two separate wick covers, used by Katz and Lewis, pieces of Perspex attached to the Perspex covering the plate (C in Figure 3-14ii), pressed on the wicks at each end, so reducing evaporation and ensuring more even contact between the wick and the layer.

Buffers of two pH values were prepared as follows: pH 5.3; ten millilitres of pyridine and four millilitres of acetic acid were made up to one litre with distilled water, (Efron, in Smith Vol. II p. 170, 1960); pH 2.0; 34 ml. of 90% formic acid and 114 ml. of acetic acid were made up to two litres with distilled water, (Bieleski and Turner, 1966). Reagents were redistilled or 'Analar'.

Wicks for the 11.5 x 11.5 cm. plates were cut from Whatman No. 1 chromatography paper and covered with dialysis tubing which had been soaked for at least 24 hr. in buffer. Wicks for 20 x 20 cm. plates contained 'Miracloth' (Calbiochem.), inside dialysis tubing.

Method

After the extract was spotted on to the plate the buffer was sprayed on. The origin was wet last by allowing the buffer to flow from the surrounding layer, so that the spot was concentrated. Excess of the buffer was blotted off the layer and the plate placed in the electrophoresis apparatus. After the wicks had been firmly pressed against the layer, the appropriate voltage was applied to the plate.

After electrophoresis, the layer was dried at room temperature with an eight inch axial flow fan and the compounds applied as a band to the plate were concentrated into spots for chromatography, with 1% acetic acid, by eluting them up to a line through the upper end of the origin. The flow of air to dry the layer was at right angles to the direction of electrophoresis.

Electrophoresis of 11.5 x 11.5 cm. plates were carried out with 500 V supplied to the elctrodes of the apparatus. This resulted in a current flow of about 10 to 12 milliamperes (mA.) for the pH 5.3 buffer and about 8 mA. for the pH 2.0 buffer. For the 20 x 20 cm. plates 1,140 volts was supplied to the electrodes and this resulted in current of about 10 mA. at pH 2.0. When 2000 volts were supplied the resulting current was about 20 mA. at pH 2.0 and 15 mA. at pH 5.3.

3-15

Chromatography

For both paper and thin layer chromatography the following solvents were used:- Butanol-Pyridine-water, BPW, (1:1:1); n-propanol-water, PW, (7:3); butanol-acetic acid water, BAW (12:3:5); Phenol-water PhW, (80:20). The following solvents were used for paper chromatography only: methanol-pyridine-water, MPW, (85:4:15); pyridine water, Pyr.W, (80:20). All proportions are in volumes. 'Analar or redistilled solvents were used.

Ascending two dimensional paper chromatography was carried out as described in Appendix 3-2, after Smith (p. 11, Vol. I, 1960). Sheets of Whatman No. 1 chromatography paper (46 x 57 cm.) were cut into 22 x 18 cm. rectangles, as four of these fitted exactly on to one piece of 43 x 36 cm. X-ray film. Extracts, after they had been spotted on the origin, were dried at room temperature. Both of the runs were carried out during the day for six to eight hours, so that the whole procedure, took two days. Solvents were prepared freshly each time, except for PW and PhW, and were evaporated after

chromatography at room temperature.

Thin layer chromatograms were developed, after the spots had been dried at room temperature, in battery jars at room temperature. The 11.5 x 11.5 cm. plates were run for two to three hours and the 20 x 20 cm. plates were run for five to seven hours, without prior equilibration. Afterwards they were dried at room temperature.

3-16

Purification of (^{35}S) methionine

(^{35}S) methionine was purified immediately before use.

Purification by ion exchange was considered first, but oxidation products of this amino acid, as well as many of the expected radiolysis products, are ionic so that the amino acid could not be separated from these expected impurities by retention on an ion exchange resin. Chromatography of methionine on paper has also disadvantages in that it is readily oxidized to the sulphoxide and sulphone and that impurities from the paper and solvent are eluted with the methionine.

To avoid some of these difficulties the paper chromatography of methionine was followed by its absorption on to a cation exchange resin. The chromatography paper was washed with EDTA before use, and thiodiglycol was dissolved in the developing solvent to prevent oxidation of methionine. These substances, as well as neutral and anionic impurities from the chromatography paper and solvent, separated from the methionine as they pass through a cation resin.

Doney and Thompson (1966) chromatographed methionine and its sulfoxide in methanol-pyridine-water (MPW) and pyridine-water (Pyr.W) as these solvents do not catalyze the oxidation of methionine. Butanol-acetic acid-water (BAW) on the other hand brings about extensive oxidation during the running and drying of the solvent.

MPW was therefore tried first as a solvent to purify methionine. After chromatography the methionine was eluted

and absorbed on Zeocarb 225 (H^+). Methionine was eluted from the resin with 2N ammonia and the ammonia was removed with a weak acid cation exchange resin, as described by Thompson et al.(1959) and further described in Appendix 3-3. Samples of the solution was chromatographed in one dimension on 11.5 x 11.5 cm. Thin layer plates with each of BAW and Phenol-water, containing 0.2% thiodiglycol. Apart from the sulphoxide an extra radioactive spot was found with an Rf greater than that of methionine.

To try to remove this contaminant, another sample of (^{35}S) methionine was further purified after chromatography in MPW by chomatography in Pyr.W. The latter solvent also failed to separate the impurity from methionine. As a result methionine used in Experiment 20 to test for the formation of possible artifacts from methionine during the extraction of sulphur compounds contained this extra compound.

After the two chromatographic runs in MPW and Pyr.W failed to completely purify methionine, BAW was used as a first solvent followed, after elution of the (^{35}S) methionine, by chromatography in MPW so that methionine sulphoxide, formed from methionine on drying the paper, was separated from the latter after chromatography in MPW. The (^{35}S) methionine was then separated by ion exchange, as before, and as fully described in Appendix 3-3.

Heise and Mittag (1965) had examined the purity of (^{35}S) methionine with BAW and Phenol water, both containing 0.2% thiodiglycol. Chromatography of the purified (^{35}S) methionine in these two solvents on 11.5 x 11.5 Thin layer plates (TL plates) showed that the solution was free from radioactive compounds whose Rf's differ from methionine in BAW and Phenol-water, except for a little sulphoxide.

3-17

Detection of compounds

Autoradiography

Ilford Gold Seal X-ray film (a fast screen type) was exposed to chromatograms and thin layer plates after radioactive ink was spotted on the corner of these, for one to four weeks as stated. These films were developed, fixed and rinsed according to the manufacturer's instructions.

Ninhydrin reagent

Paper chromatograms and 11.5 x 11.5 cm. TL plates were dipped in 0.2% ninhydrin in acetone containing 2% pyridine, after Smith (Vol. I, p. 95, 1960). 20 x 20 cm. plates were sprayed with the reagent which contained 2% collidine in place of pyridine. After treatment, plates and papers were heated at 80°C. for a few minutes to develop the colour. Thin layer plates (20 x 20 cm.) used for the identification of compounds were left one day at room temperature for the colour to develop.

Iodine Azide reagent:- to detect thiol and disulphide groups

This was prepared as described by Smith (1960). Equal volumes of 1.27 g. iodine in 100 ml. of ethanol and 3.25 g. sodium azide in 25 ml. of water plus 75 ml. of ethanol, were mixed and immediately sprayed on to the thin layer.

Iodoplatinate reagent:- to detect thioethers

The reagent as modified by Njaa (1963) to contain starch was made from the following solutions immediately before use: 0.1 ml. of 10% chloroplatinate, 7 ml. of 1.1% potassium iodide solution and 7 ml. of water. The mixed solution was added to 80 ml. of 0.5% starch solution and sprayed on to the chromatograms. Methionine on paper chromatograms gave a stable blue colour, but on a thin layer plate the colour rapidly faded.

3-18

Assay of Radioactivity

To measure the quantity of radioactivity in a solution, a small portion was pipetted on to a planchet and spread to about one centimeter in diameter with a few drops of ten per cent isopropanol. The resulting solution was dried under an infra-red heat lamp and the radioactivity counted with a thin window Geiger-Müller tube as described in Appendix 4.

Counts recorded were corrected for background, and decay from the day on which the experiment was carried out, but not for absorption which was assumed to be insignificant.

The radioactivity of the solutions in Experiment 23 was measured in Bray's scintillation solution, (1960) as described in that Experiment and in Appendix 4.

3-19

Preparation of standards

Sulphur amino acids were oxidized as described by Peterson and Butler (1962).

Sulphoxides of methionine, S-methyl-cysteine and cystathionine were prepared from the thioethers by oxidation with three per cent w/v hydrogen peroxide. Sulphones derived from methionine and S-methyl-cysteine, and the sulphonic acids of cystine, homocystine and glutathione were prepared by oxidation of the parent compounds with three per cent hydrogen peroxide and 0.02% ammonium molybdate. After oxidation, as detailed in Appendix 3-4, the samples were freeze dried and taken up in ten per cent isopropanol.

Acetamido derivatives of cysteine, glutathione and homocysteine were prepared as detailed in Appendix 3-4. Acetamido-cysteine (ACys) and acetamido-glutathione (AGl) were purified by ascending paper chromatography. Acetamido-homocysteine (AHCys) could not be freed from homocystine by paper chromatography or thin layer electrophoresis. It was however possible to partially separate these two compounds by electrophoresis at pH 2.0, so the presence of AHCys was

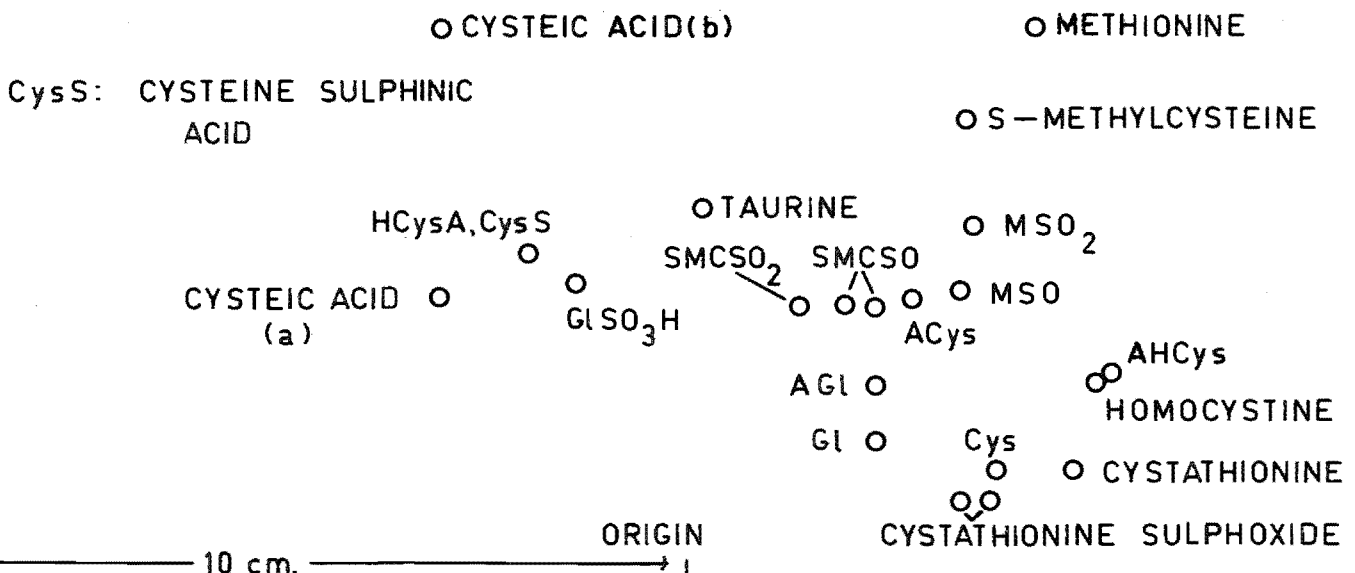
FIGURE 3-19

THE RELATIVE POSITIONS OF SOME SULPHUR COMPOUNDS ON A TL PLATE

+ve ← ELECTROPHORESIS AT pH 2.0 (1,140V, 35 min.) → − ve

PW ↑

ACys: ACETAMIDOCYSTEINE
AGl: ACETAMIDOGLUTATHIONE
AHCys: ACETAMIDOHOMOCYSTEINE
Cys: CYSTINE
Gl: GLUTATHIONE (major spot only)
$GlSO_3H$: GLUTATHIONE SULPHONIC ACID
MSO: METHIONINE SULPHOXIDE
MSO_2: METHIONINE SULPHONE
SMCSO: S-METHYLCYSTEINE SULPHOXIDE
$SMCSO_2$ S-METHYLCYSTEINE SULPHONE
(b) Rf AFTER ELECTROPHORESIS.



shown by spraying with ninhydrin, Iodine-azide and the iodo-platinate reagent, replicate runs of the oxidized and unoxidized AHCys preparation.

Preparation of a map of some standards

The compounds were separately electrophoresed and chromatographed in one dimension only. Methionine was used as a standard for the comparison of Rf values so that the positions in the two dimensional picture (Figure 3-19) are relative. However it was found that cysteic acid added to an extract had an unexpected Rf in PW, after electrophoresis at pH 2.0. On the other hand AHCys added to the same extract migrated to its expected position so that only the mobility of acidic compounds was likely to be affected.

3-20

Identification of Radioactive Sulphur Compounds

Some of the radioactive compounds were tentatively identified by comparing their positions with those of some expected sulphur metabolites which had been separated by electrophoresis and chromatography (Figure 3-19). Most of these compounds were then further identified by eluting from the layer and co-chromatographing with a known compound.

For elution of compounds from the thin layer, their positions were located as follows. The outline of each spot on the autoradiogram was traced onto a piece of transparent paper, which was located on the thin layer plate by using the radioactive ink spots. The traced outline of each spot was then pricked through the paper onto the layer with a needle.

To hold the thin layer while it was eluted, Pyrex glass capillaries 1-2 mm. in diameter were drawn out and broken off into 5-6 cm. lengths. These were plugged with non-absorbent cotton wool about one centimetre from an end.

Each of the outlined spots was scraped off with one of the glass capillaries and sucked up against the cotton wool so that the scrapings from the layer formed a small column.

To elute the compound for co-chromatography, four to six microlitres of a solution of the standard (4-6 µg. of the compound) were allowed to percolate through the thin layer in the capillary, after which the first 2 µl. were spotted onto a thin layer plate. If the radioactive compound alone was required, it was eluted with distilled water.

After electrophoresis or chromatography of the compounds, an X-ray film was exposed for 9 days to the thin layer plate, which was later sprayed with ninhydrin. The shape and outlines of the spots detected by the ninhydrin spray and those on the X-ray film were compared to see whether they were concurrent.

3-21

Tentative identification of some sulphur containing compounds

Since the separation of the neutral fractions from the extracts was at pH 2.0 followed by PW chromatography, the identification was carried out in pH 5.3 buffer and BPW; in case two compounds coincided after the two-way separation by pH 2.0/PW. BPW was chosen because of suitability for the separation of sulphur amino acids (Peterson and Butler, 1962). Electrophoresis at pH 5.3 separated a number of the sulphur compounds present in the tomato roots extracts (Figure 3-11iii).

As the pattern of spots on pH 2.0/PW TL plates was similar for the different treatments, it was assumed that those spots present at similar positions on the different plates were identical compounds. One representative spot of all those thought to be the same was chromatographed in BPW and another representative spot was eluted for electrophoresis.

Methionine sulphoxide (MSO), methionine sulphone (MSO_2) and acetamido-cysteine (ACys) were identified by co-chromatography and co-electrophoresis with standards. Some of the methionine sulphoxide was oxidized to methionine sulphone during its elution and spotting on the plate. Several breakdown products from methionine sulphoxide were also present but these were not ninhydrin positive. The compound Y was identified as a degradation product of acetamido-cysteine (ACys) from its

similar mobility at pH 2.0 to one of the two extra radioactive compounds present in eluted ACys. On oxidation with 3% hydrogen peroxide Y formed another compound with similar mobility at pH 2.0 to the other degradation compound.

Methionine was identified, after oxidation to methionine sulphoxide, by co-chromatography and co-electrophoresis; as well as by the correspondence of added carrier methionine to radioactive methionine after pH 2.0/PW separation of an extract. Acetamido-glutathione (AGl) was identified from its electrophoretic mobility in pH 2.0 and 5.3 buffer and its appropriate Rf in PW. Its mobility in pH 2.0 buffer was unaffected by oxidation with hydrogen peroxide. Acetamido-glutathione alone, of all the sulphur compounds identified, was ninhydrin positive.

Neither acetamido-homocysteine nor cysteic acid could be detected in the pH 2.0/PW separations of any of the neutral amino acid fractions. Their positions were determined from adding the standard to an extract.

Sulphate present in the neutral fractions was identified by elution and electrophoresis with added (^{35}S) sulphate at pH 5.3 followed by chromatography in PW. Sulphate in the HCl fractions was identified from its strong anionic properties during ion exchange and the failure of added (^{35}S) sulphate to produce an extra spot in pH 5.3/PW separations of the HCl fraction, as well as its position on the TL plate.

Experiment 1

The choice of a medium for the growth of Petkus rye roots

I. The inorganic component

Preliminary work in this laboratory has suggested that tomato roots grew better on Bonner's (1940) medium with Charles and Street's micronutrients than they did on Street's medium (Boll and Street, 1951). So the growth of rye roots on modified Bonner's was compared with that on Charles and Street's medium (1959) to find out which was the better medium for their growth. At the same time it was decided to compare these two media with Murashige and Skoog's (1962) medium, on which sunflower pith cultures had grown well in this laboratory, since the latter contained very much higher concentrations of nutrients than either modified Bonner's or Charles and Street's medium.

The growth of root tips excised from rye seedlings was compared in these three media, because insufficient clonal roots were available. The media were prepared as set out below and inoculated with tips from seeds germinated and sterilized as described in Methods and Materials.

	Increase of main axis (mm.)	lateral number	lateral length (mm.)	No. harvested
modified Bonner's (III)	90 ± 13	6.6 ± 1.5	90 ± 13	19
Charles and Street's (I)	55 ± 7	6.2 ± 1.7	63 ± 22	17
Murashige and Skoog's (II)	43 ± 7	12.1 ± 2.5	81 ± 18	19

The growth of Petkus rye tips derived from seedlings in several inorganic media, all containing 2% glucose, 30 ppm yeast extract and Streets' vitamins.

From the results of this it was concluded that growth, expecially of the main axis, was better in modified Bonner's medium. In Murashige and Skoog's medium, growth of seedling tips was very poor but it was considered that this might have

		Increase of main axis (mm.)	Lateral number	Lateral length (mm.)	No. harvested
Modified Bonner's (III)	P1	38.9±1.6	14.1±1.0	175±32	10
	P2	38.1±0.7	11.6±1.0	232±15	10
Charles & Street's (I)	P1	40.9±2.0	19.1±1.0	151±22	9
	P2	44.3±1.9	20.2±1.4	209±29	10
Murashige & Skoog's (II) Autoclaved	P1	27.5±1.0	12.9±1.3	69±11	8
	P2	21.9±2.3	11.9±0.9	55±5	9
Filter Sterilized	P1	30.8±0.7	15.8±1.1	122±18	10
	P2	33.7±2.0	15.0±1.5	109±17	10

Table 4-1

The growth of Petkus rye roots in several inorganic media, all containing 2% glucose, 30 ppm yeast extract and Street's vitamins. P1 and P2 refer to passage one and passage two respectively.

been due to a white precipitate, formed during autoclaving.

When the growth rates of clonal tips in these three media were later compared, a treatment of filter-sterilized Murashige and Skoog's medium was included in addition to the autoclaved medium, to establish whether the formation of the precipitate was solely responsible for the poor growth of the tips.

Experimental details

These three media (I, II and III) were prepared as described in Appendix 1 and Methods and Materials 3-2, 3-5 and 3-6. All contained two per cent glucose, Street's vitamins and 30 ppm yeast extract, which was the concentration used by Roberts and Street (1955).

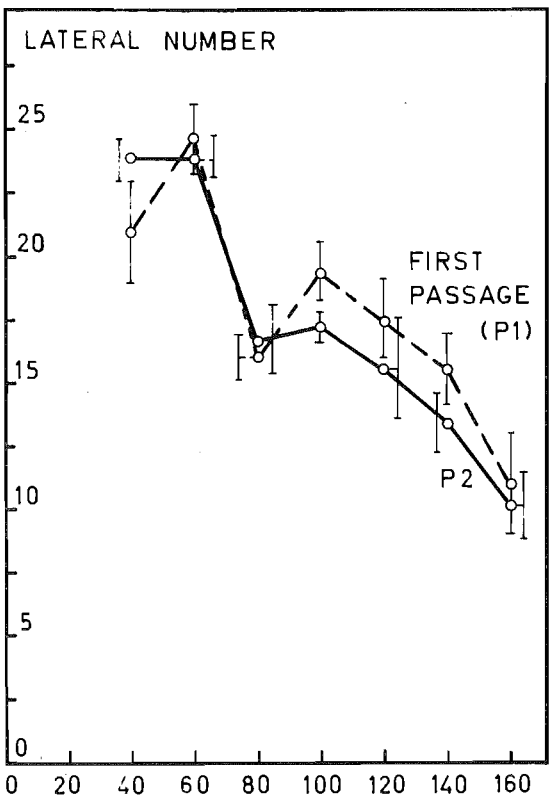
The pH of the media after autoclaving or filter sterilization was 4.4.

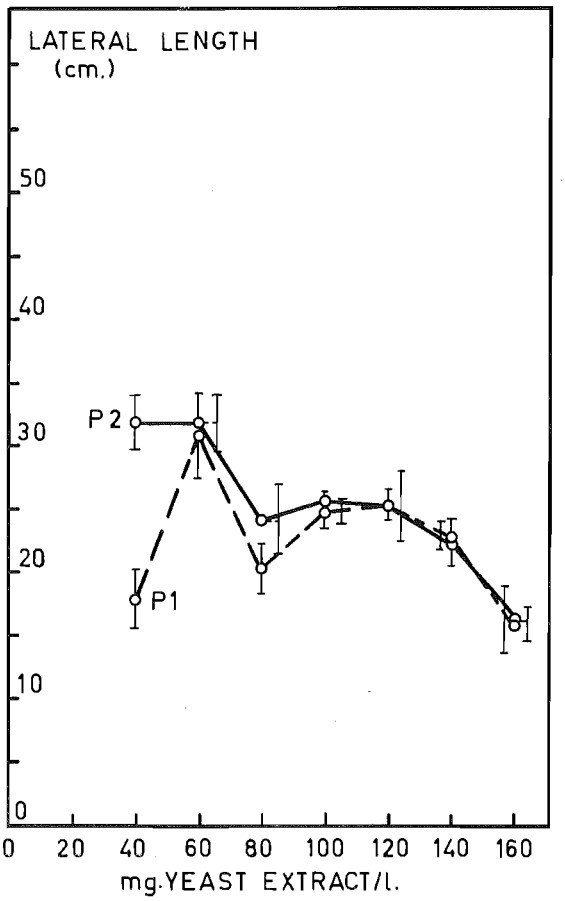
Results and Discussion

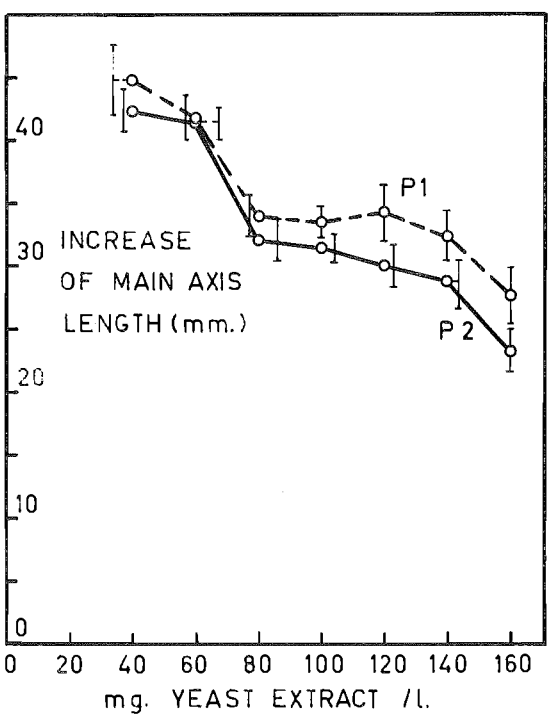
Table 4-1 shows that the increase of main axis length and lateral length in Murashige and Skoog's medium, either filter sterilized or autoclaved, was much less than that in either modified Bonner's or Charles and Street's media, although filter sterilizing the first medium compared with autoclaving enhanced the increase of main axis and lateral length. For modified Bonner's and for Charles and Street's medium, the increase of main axis and the lateral length measurements were similar for both passages, although the lateral number was greater in Charles and Street's medium.

It was concluded from this that modified Bonner's medium and Charles and Street medium allowed similar growth rates for rye roots. The greater lateral number in Charles and Street's medium was brought about by the production of many more short laterals nearer the tip in that medium but this difference was not important as it contributed little to the bulk of the roots. The lateral length was slightly greater in modified Bonner's medium, although not significantly so, and it was therefore subsequently used for the growing of rye roots, and was referred to as 'rye clone' medium. Later in this work it was modified again.

FIGURE 4-1







THE GROWTH OF RYE ROOTS IN CHARLES AND STREET'S MEDIUM WITH VARYING CONCENTRATIONS OF YEAST EXTRACT.

II. The choice of a concentration of yeast extract

The Petkus rye tips inoculated into the first part of this experiment seemed to grow slowly for the first five days. Thirty parts per million of yeast extract might have been too low a concentration for optimum growth since rye tips had grown much more slowly in 25 ppm than in 250 ppm yeast extract, (Establishment of a clone). Growth of the tips was therefore compared in a series of concentrations of yeast extract ranging from 40 ppm (as 30 ppm seemed to be too low for rapid growth) to less than 250 ppm since this concentration inhibited growth of the clone after several subcultures, (Materials and Methods 3-9).

Experimental details

To Charles and Street's medium (I) was added yeast extract to give the concentrations shown below. After the media were autoclaved the pH was 4.4.

Medium (I) plus 40, 60, 80, 100, 120, 140, 160, ppm YE.

Results and Discussion

Figure 4-1 (Appendix Table 5-1) shows that the lateral length in both passages increased as the yeast extract concentration was raised from 40 to 60 ppm and then fell with a gradually increasing rate of descent. The increase of the main axis was less for concentrations of yeast extract above 40 ppm and the lateral number also fell quite rapidly over the same concentrations.

Thus 60 ppm was the best of the yeast extract concentrations used and the growth of tips with this level of yeast extract was satisfactory over two passages.

After this Experiment was completed, the clone appeared to be slightly less vigorous than before so the concentration of yeast extract in the medium (Establishment of a clone) was also reduced to 60 ppm.

III. The replacement of yeast extract with tryptophane in experimental media

The results of the second part of this Experiment showed that 60 ppm yeast extract was optimum for growth. Yeast extract contains a range of amino acids, including methionine, and other organic compounds, so that its use for experimental media would have made growth of tips on sulphur compounds difficult to interpret. Roberts and Street (1955) found that tryptophane would replace yeast extract as a growth factor for Petkus rye II. The variety of Petkus rye used in this work was different, and growth of roots with 60 ppm yeast extract was compared with growth with 0.54 ppm tryptophane, which Roberts and Street found to be equivalent. Only this one concentration was compared as Roberts and Street showed that the response curve of roots to tryptophane parallelled that of yeast extract.

Experimental details

To 'rye clone' medium (III) was added 60 ppm yeast extract or 0.54 ppm tryptophane. These additions were autoclaved with the bulk of the medium since Roberts and Street found that tryptophane was active only after autoclaving.

Results and Discussion

		Increase of main axis (mm.)	t for 18° F	Lateral number	t for 18° F	Lateral length (mm.)	No.
60 ppm YE	P1	39.2±1.8	3.44*	13.8±0.8	2.9*	237±28	10
0.54 ppm Tryptophane	P1	57.1±3.1		19.1±1.6		241±25	10
60 ppm YE	P2	35.5±1.2	2.9*	12.6±0.8	2.9*	245±20	10
0.54 ppm Tryptophane	P2	43.1±2.3		16.2±1.0		257±27	10

The growth of rye tips in 'rye clone' medium containing either yeast extract or tryptophane.

The table shows that the lateral lengths of roots was similar whether grown with tryptophane or with yeast extract. Tryptophane-grown roots had a significantly greater increase of main axis length and number of laterals ($p = 0.05$) than roots grown with yeast extract. Although the growth pattern in these two treatments was dissimiliar, perhaps because of the variety of compounds present in yeast extract, tryptophane supplied sufficient of the growth requirements of rye roots to maintain growth, as measured by lateral length, over two passages. This amino acid was therefore used in place of yeast extract in later experiments with rye.

Experiment 2

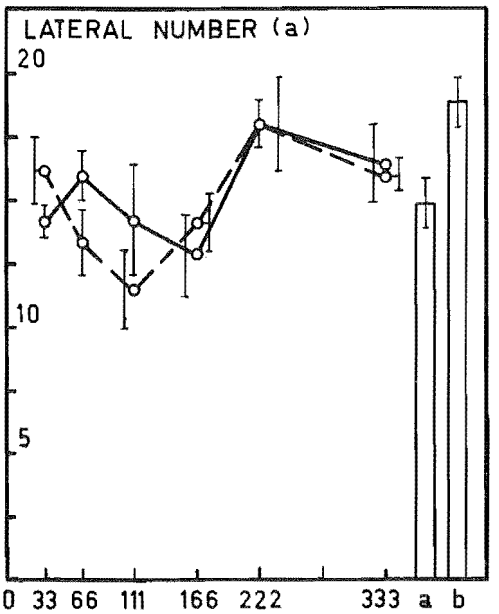
The growth of rye roots with several levels of nitrate at two sulphate levels.

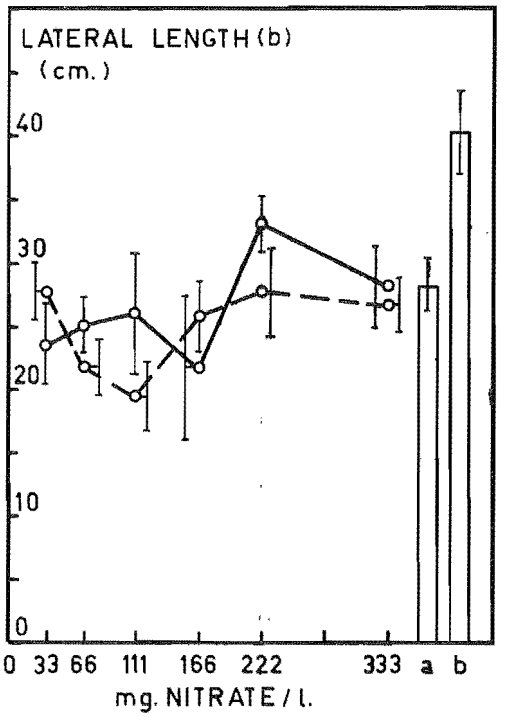
Nitrogen and sulphur are similar in that their anions are reduced by six electrons overall when assimilated into organic compounds. Further an enzyme reducing both sulphite and hydroxylamine - a possible intermediate in the reduction of nitrate - has been isolated from Neurospora (Siegel, Leinweber and Monty, 1965). If the elements nitrogen and sulphur share some part of their assimilatory pathways in this way, they could, under certain circumstances, compete with one another.

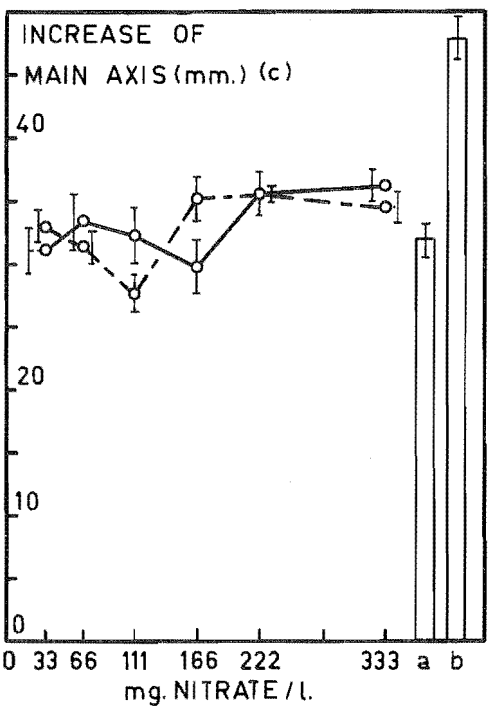
The growth of rye roots with several concentrations of nitrate at two concentrations of sulphate was measured to try to establish whether growth varied linearly with the concentration of one, when its supply was alone limiting growth. Now interactions occur between ions during uptake, e.g. potassium and sodium, but nitrate and sulphate were reported by Leggett and Epstein (1956) to be independently taken up into barley roots. It could therefore be assumed that if an interaction did occur between them, it indicated a particular relationship between their assimilatory pathways.

The concentrations of sulphate and nitrate used by Bonner (1940) were assumed to be near optimum (these concentrations are referred to as standard (S) concentrations e.g. X1S), and other concentrations were chosen around these. A miscalculation was made in the concentration of sodium nitrate required to replace calcium nitrate and potassium nitrate so that the intended X1.5 S N concentration (166 mg. nitrate/l.) became almost the standard concentration and the 111 mg. nitrate/l. concentration - intended as X1S was used to compare growth in magnesium sulphate with that when this salt was replaced with magnesium chloride and sodium sulphate.

FIGURE 4–2i







THE GROWTH OF RYE ROOTS DURING THE FIRST PASSAGE WITH VARYING LEVELS OF NITRATE AT TWO LEVELS OF SULPHATE.

HISTOGRAMS: 111 mg. NITRATE /l.; 14 mg. SULPHATE /l. ADDED AS
(a) SODIUM SULPHATE,
(b) MAGNESIUM SULPHATE.

CURVES: o——o 7 mg. SULPHATE/l.
o- - -o 21 mg. SULPHATE /l.

Experimental details

To 'rye clone' medium (III) containing 0.54 ppm tryptophane in place of yeast extract, with potassium nitrate, calcium nitrate and magnesium sulphate replaced with their respective chlorides were added sodium nitrate and sodium sulphate to give the concentrations shown below. A further treatment of 'rye clone' medium, containing 0.54 ppm tryptophane, with potassium and calcium nitrates replaced with their chlorides but containing magnesium sulphate was prepared to see whether the addition of extra sodium and chloride ions affected growth. The pH of the media was 5.0.

Media as above contained the following additions:

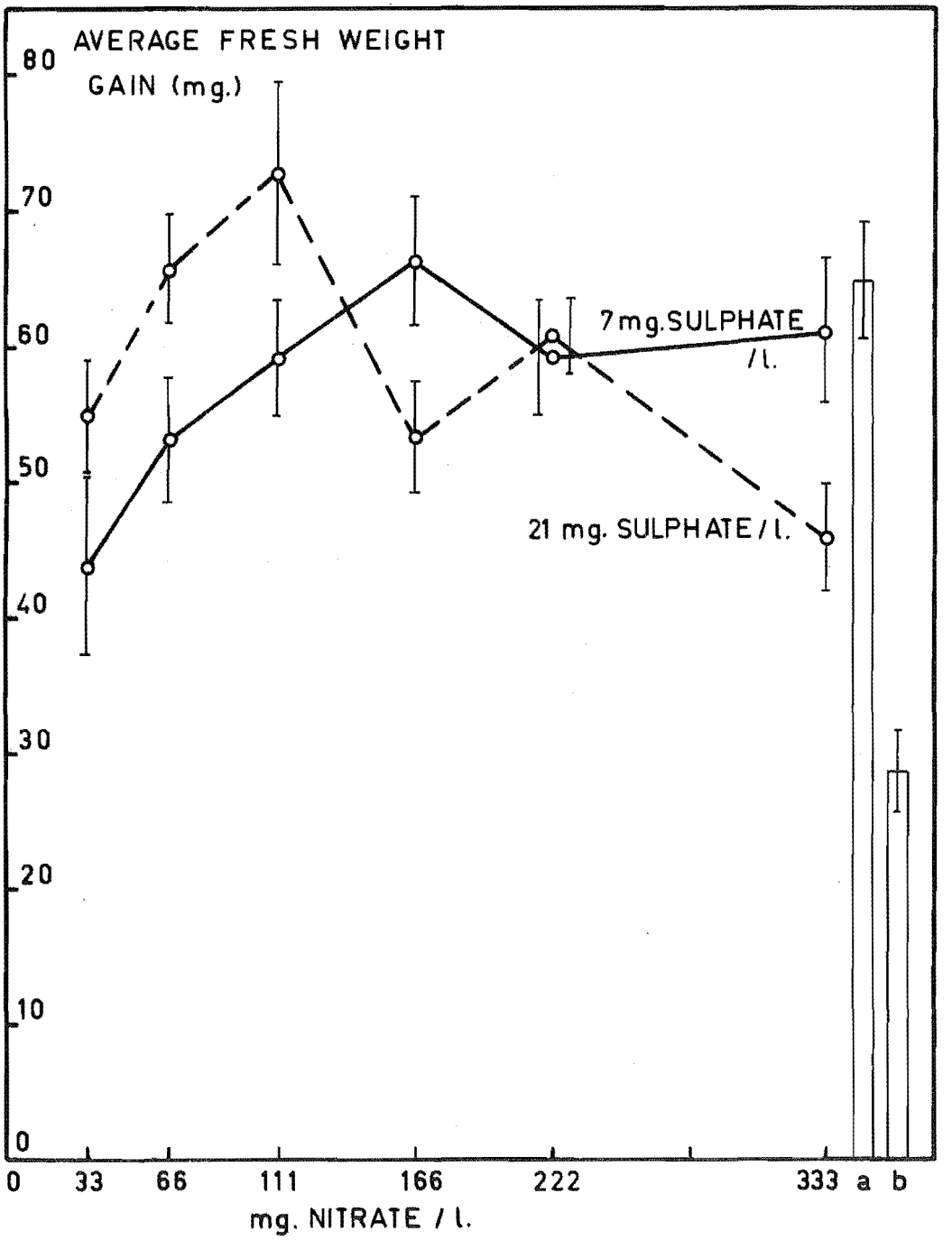
mg./l. sulphate as sodium sulphate	mg./l nitrate as sodium nitrate					
7	33	66	111	166	222	333
21	33	66	111	166	222	333
14	-	-	111	-	-	-
mg./l. sulphate as magnesium sulphate						
14	-	-	111	-	-	-

Results and Discussion

Figure 4-2i (a), (b) and (c), (Appendix Table 5-3) show that growth in the first passage was unaffected by the level of either sulphate or nitrate except that lateral length and increase of main axis were greatest in the magnesium sulphate containing treatment. The large number of secondary laterals (these are the laterals formed on laterals off the main axis) in treatments containing added magnesium chloride contributed considerably to the total mass of plant material (Figure 3-7) although they were not measured. Therefore in the second passage, growth was measured by the fresh weight gain, as described and discussed in Methods and Materials 3-7.

To establish whether a decrease of lateral length occurred in the second passage as it did in the first when extra chloride ions were added in the magnesium chloride, length measurements

FIGURE 4—2 ii



THE GROWTH OF RYE ROOTS DURING THE SECOND PASSAGE WITH VARYING LEVELS OF NITRATE AT TWO LEVELS OF SULPHATE. HISTOGRAMS: 111 mg. NITRATE / l.; 14 mg. SULPHATE / l. ADDED AS (a) SODIUM SULPHATE, (b) MAGNESIUM SULPHATE.

were made for the magnesium sulphate containing treatment and one of the magnesium chloride treatments - the one with the lowest concentration of nitrate and sulphate.

Figure 4-2ii (Appendix 5-3) shows that growth of Petkus rye roots in the second passage, as measured by fresh weight and dry weight, was generally better at levels of nitrate below 111 mg/l with 21 mg/l sulphate than with 7 mg/l. sulphate. While this result was in contradiction to that in Experiment 3 where omitting sulphate had little effect. This difference could be ascribed to the alteration of ion concentrations when sulphate is varied. Increasing levels of nitrate from 33 to 111 mg./l. increase growth up to an optimum at 111 mg. nitrate. At higher levels of nitrate growth declined although considerable variability made this result only tentative. When magnesium chloride and sodium sulphate replaced magnesium sulphate growth was much greater as shown in Figure 4-2ii.
That this was not just a feature of the second passage was shown by the similar increase for both passages of total length, lateral length and lateral number for roots in the magnesium sulphate treatment compared with the roots in 7 mg. sulphate/l. - 33 mg. nitrate/l. treatment.

Medium as described in text	Second passage Main axis increase (mm.)	Lateral number	Lateral length (mm.)
treatment with added magnesium sulphate	45.9 ± 1.8	19.4 ± 1.0	346 ± 20
treatment with 7 mg. sulphate/l. and 33 mg. nitrate/l.	29.6 ± 2.8	14.0 ± 1.1	218 ± 16

Further the increased number of secondary laterals is a feature of both passages.

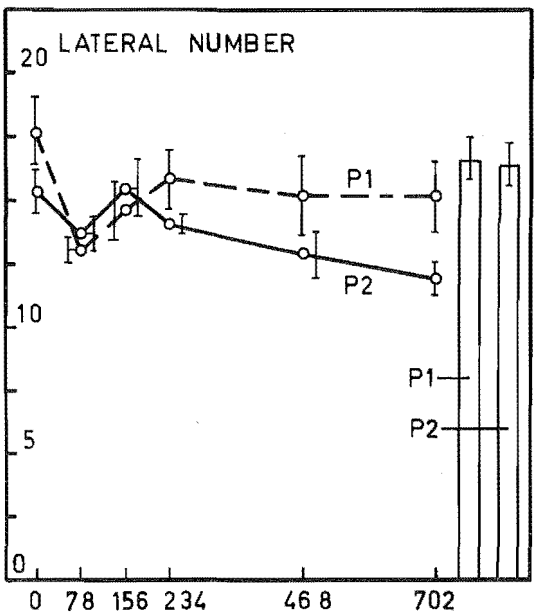
It appeared that the level of nitrate has no effect upon sulphate assimilation, since the growth increase was linear with the increasing nitrate concentrations. As all the amounts of nitrate added were very much more than sufficient for growth, the possibility that this decrease of growth was

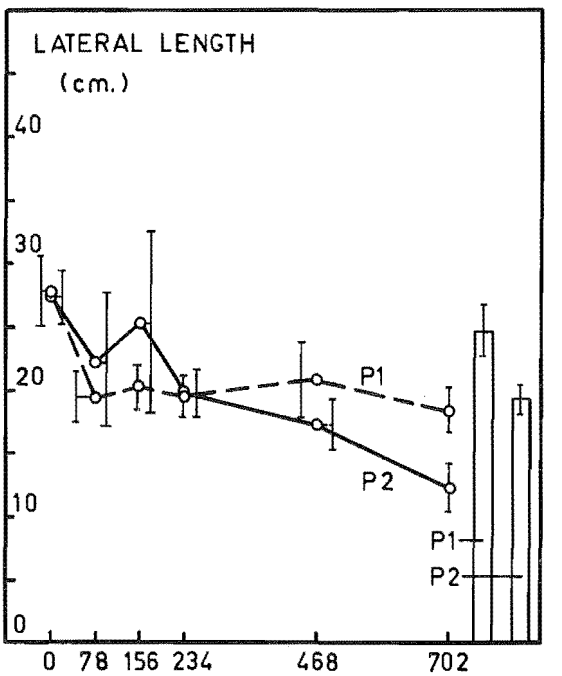
brought about by nitrate deficiency is unlikely unless the rate of nitrate transport was limiting at the lower nitrate concentrations. This effect of nitrate concentration does not appear to be related to the level of sulphate at the concentrations shown and it was concluded that no further investigation of sulphur nutrition in relation to nitrogen nutrition was justified from these results.

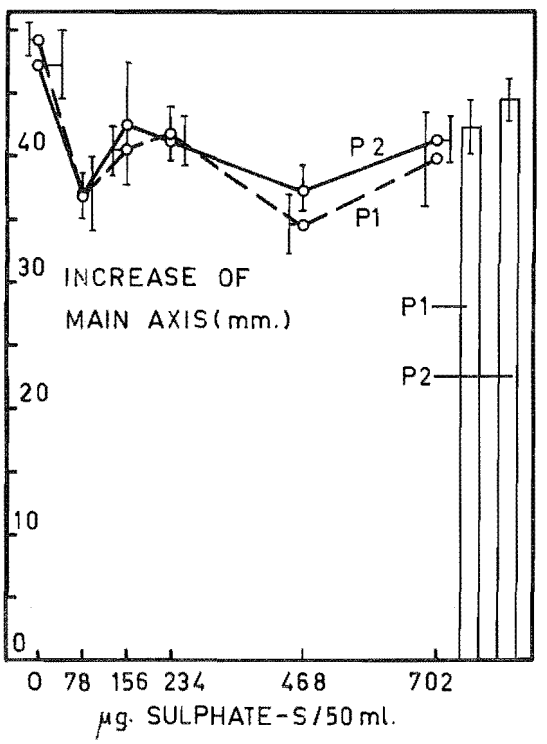
Since 111 mg. nitrate/l. (X0.65) was sufficient for optimum growth, this level is used in further experiments with rye.

'Rye clone' medium (III) modified by replacing magnesium sulphate with magnesium chloride and sodium sulphate, was used in future experiments since growth of rye roots was greater in this variant and the level of sulphate contamination based on the manufacturer's maximum limits was lower: calcium chloride was available as an 'Analar' salt whereas calcium nitrate was not. This new variant was described as 'tryptophane' medium (IV).

FIGURE 4-3







µg. SULPHATE-S/50 ml.

THE GROWTH OF RYE ROOTS IN 'RYE CLONE' MEDIUM CONTAINING TRYPTOPHANE WITH VARYING AMOUNTS OF SODIUM SULPHATE.

HISTOGRAMS: MAGNESIUM SULPHATE ADDED TO THE MEDIUM AT 234 µg. SULPHATE-S /50 ml. MEDIUM.

Experiment 3

The growth of Petkus rye roots with several levels of sulphate

The growth response of rye roots to increasing sulphate concentrations was required as a standard of comparison with their response to sulphur compounds. The sulphate concentration used by Bonner (1940) which was less than that used by Charles and Street (1959) was initially assumed to be near the optimum for the growth of tomato roots and thus probably near the optimum for rye roots. Several concentrations around this level were chosen, and among them was included a sulphate - omitted treatment to test for the presence of a sulphur impurity in the medium.

Experimental details

To 'rye clone' medium (III) containing 0.54 ppm tryptophane, with the magnesium sulphate replaced by magnesium chloride at equivalent concentration, was added sodium sulphate to give the concentrations shown below. A treatment of 'rye clone' medium (III), with 0.54 ppm tryptophane, containing magnesium sulphate was included to show whether the addition of sodium and chloride ions altered the growth rate of the roots.

'rye clone' medium containing magnesium chloride and 0.54 ppm tryptophane with 0, 70, 140, 234, 468, 702 µg. sulphate-S/50 ml.

'rye clone' medium plus 0.54 ppm tryptophane 234. µg.S/50 ml. as magnesium sulphate.

Results and Discussion

Figure 4-3 (Appendix Table 5-2) shows that increasing the sodium sulphate concentration did not affect the growth of the main axis or the lateral number, but reduced the lateral length slightly at higher concentrations. The replacement of magnesium sulphate with equivalent magnesium chloride and sodium sulphate had no effect upon growth. Further the omission of sodium sulphate did not limit growth in the slightest, and so sufficient sulphur for optimum growth was present as an impurity in the medium. To decide

Table 4-3

Evaluation of the quantity of sulphate supplied by the salts of the nutrient medium.

'Rye Clone' medium (III)

	$\mu g.SO_4/l.$	
KCl	1.9	
KH_2PO_4	2.0	
$Ca(NO_3)_2 6H_2O$	47.0	
KNO_3	4.0	
$MgCl_2 6H_2O$	1.48	
	56.6	which is 1 µg.S/50 ml. medium

'Tryptophane' medium (IV)

KCl	3.7	
KH_2PO_4	2.0	
$MgCl_2 6H_2O$	1.5	
$NaNO_3$	7.6	
$CaCl_2 6H_2O$	10.9	
	27.8	which is 0.4 µg.S/50 ml. medium
Glucose	1400	which is 23 µg.S/50 ml. medium

'High magnesium' medium (VIII) 1 µg.S/50 ml. medium total.

whether the sulphur contamination originated from the tip or the medium, the sulphate - omitted treatment was carried on for a further two passages beyond the two which had been planned for the experiment. As the growth rate was undiminished during these four passages, as shown by the following Table, it was concluded that the source of the impurity was the medium.

'Rye Clone' medium (III) with 0.54 ppm tryptophane

Containing magnesium chloride 0 µg. sulphate-S/50 ml.		Increase of main axis (mm.)	Lateral number	Lateral length (mm.)	No.
	P1	49.2±1.4	17.6±1.4	278±29	10
	P2	47.2±2.7	15.3±0.9	274±22	10
	P3	49.6±2.4	20.2±1.2	333±15	10
		Increase of fresh weight (mg.)			
	P3	24.4±2.1			10
	P4	16.8±1.2			10

In Table 4-3 is shown a list of the salts and sugar present in the medium, the quantities added and the maximum amount of sulphate contamination from the manufacturer's claims which was provided by each. Of these, glucose contained the greatest quantity of sulphate, providing 23 µg. S to each 50 ml. of medium. As the roots weighed about 3 mg. each when dry and probably contained around 0.1 per cent to 1 per cent of the dry weight as sulphur (Gilbert, 1951), each root required around 3 to 30 µg. S for optimum growth. The range 0.1 per cent to 1 per cent of the dry weight was reported by Gilbert (1951) to contain the sulphur contents of higher plants. Although he may not have included tomato, there was no reason to suppose it would differ very much, especially as he considered that the sulphur content was usually at the lower end of the range and only plants such as the crucifers, which contained large quantities of non-protein

sulphur compounds, had sulphur contents at the upper end of the range. From these considerations the glucose alone provided sufficient sulphate for the requirements of the roots and therefore purification of the 'Analar' glucose was indicated.

Suitable methods of removing sulphate were: firstly, precipitation as the insoluble barium salt; secondly, ion-exchange; and thirdly, recrystallization of the glucose. Precipitation was impossible as the solubility of the barium sulphate exceeded the quantity of sulphate present since, with a practicable excess of barium, 'Analar' glucose is free from visible turbidity when tested with barium chloride. Crystallization of the glucose from water was attempted by evaporating the solution under reduced pressure to avoid forming a caramel, but the solution evaporated to a sticky brown solution. Purification by ion-exchange offered a solution as sulphate was reported by Wheaton and Bauman (1951) to be among the more strongly retained anions on a strong base resin.

Experiment 4

Achieving a sulphur deficiency

The effect of ion exchanging glucose was examined in this experiment. At the same time several glucose concentrations below 2 per cent were tried to decide whether the roots grew as well on a lower concentration, which would add a smaller sulphate impurity to the medium than 2 per cent glucose.

The sulphate-chromate complex which formed in chromic acid cleaning mixture could have contributed to the sulphate impurity since it is strongly adsorbed to glass (White 1943). After cleaning the flasks with this chromic acid mixture, some of them were treated with concentrated hydrochloric acid to displace the sulphate complex from the glass, so that a comparison could be made with flasks containing the complex.

Experimental details

An anion exchange resin, De Acidite FF, 25-50 mesh 8% DVB, was purified as described in Methods and Materials 3-5 and 25 ml. poured into a 25 ml. burette. This volume was chosen so that the total exchange capacity was considerably in excess of the possible sulphate and sulphite content of the glucose, but when a larger column was prepared the volume of resin was estimated as set out in Methods and Materials 3-5.

The resin was regenerated with 100 ml. 2 N sodium chloride and washed with approximately 500 ml. of distilled water until free of chloride ion, as shown by the silver nitrate test. Glucose was passed through the column as 30 per cent w/v solution and that remaining in the column was washed out with 50 ml. distilled water. This was used in 'tryptophane' medium (IV) minus sulphate. Sodium sulphate was added to one treatment of ion exchanged glucose at 234 µg. S/50ml. The treatment with 2 per cent untreated glucose was to compare its growth with that with ion exchanged glucose as well as with lower sugar concentrations.

Flasks treated with hydrochloric acid were coated on the inside with concentrated hydrochloric acid and rinsed with tap water, single distilled water and then double distilled water.

Table 4-4

'Tryptophane' medium (IV)			Fresh weight gain (mg.)	No. of roots
with Ion exchanged glucose minus sulphate	Flasks chromed Flasks cleaned NaOH	P1	11.7±1.1	9
		P2	3.7±1.0	9
		P3	6.2±1.1	7
		P4	6.3±1.9	6
	Flasks chromed rinsed HCl	P1	9.2±0.9	10
		P2	2.6±0.3	9
	Flasks cleaned NaOH	P3	4.5±0.7	9
		P4	3.6±0.5	9
plus sulphate 234 µg.S/50 ml.	Flasks chromed	P1	20.0±4.3	8
		P2	10.7±1.1	8
		P3	35.0±4.4	7
		P4	33.0±4.9	10
with untreated glucose all flasks chromed	Sugar concentration (per cent)			
	2	P1	16.0±1.2	9
		P2	10.0±1.0	8
		P3	26.2±2.1	7
		P4	26.5±3.6	9
	1.5	P1	12.5±1.8	9
		P2	4.5±1.2	9
	1.0	P1	9.1±1.1	10
		P2	1.4±0.2	10
	0.5	P1	7.1±0.8	10
		P2	2.3±0.5	9

Two passages of 14 days duration were planned, but during the second when growth of the minus sulphur treatments continued, it was decided to continue the experiment for a further two passages to see whether the roots would die.
The media for the third and fourth passages were made up as described above except that flasks intended for the minus sulphate treatments were cleaned with alcoholic sodium hydroxide instead of chrome and hydrochloric acid.

Untreated glucose was added to 'tryptophane' medium (IV) to give the concentrations shown below.

The pH of all the media after autoclaving was 4.3.

'Tryptophane medium (IV)

with 2% ion exchanged glucose µg.S/50 ml.	with untreated glucose and 234 µg.S/50 ml. glucose (per cent)
0 (2 treatments)	2
234	1.5
	1.0
	0.5

Results and Discussion

Table 4-4 shows that growth of Petkus rye roots was less in any of the glucose concentrations below 2 per cent, and so, even if a concentration between 1.5 per cent and 2 per cent was optimum, no worthwhile reduction of the sulphur impurity could have been achieved by reducing the sugar concentration alone.

Table 4-4 shows that the ion-exchanging of the glucose did not affect the growth of Petkus rye roots when compared with untreated glucose in plus sulphate medium. When sulphate was omitted from the medium with ion-exchanged glucose there was a decrease of growth, but the treatment in which the flasks were rinsed with hydrochloric acid had only slightly less growth than the other minus sulphate and this was not significant. Although growth fluctuated from week to week, all the treatments tended to increase or decrease together.

The minus sulphate roots continued growing at an apparently undiminished rate in the fourth passage, so the ion-exchanging

of the glucose depleted the sulphur impurity of the medium sufficiently to reduce growth but not sufficiently to cause death of the tips. This indicated that there was still a considerable sulphur impurity present.

It appeared that sulphate complexes absorbed to glass were not an important source of sulphur as the growth of the two minus sulphur treatments remained the same relative to each other after all flasks were cleaned with alcoholic sodium hydroxide for the third and fourth passages and had differed only slightly.

Experiment 5

The affect of initial pH upon the growth of rye roots

If the growth of the roots during a passage was rapid the pH drifted almost to neutrality. The affect of initial pH upon the growth rate was therefore tested to determine whether this pH change during growth was beneficial. To try to prevent this change, in case it was deleterious, ammmonium nitrate was used instead of sodium nitrate as a 'physiological buffer', and in another treatment the phosphate concentration was increased five times to add to the buffering capacity of the medium.

Experimental details

A change of pH from around 5.0 to 4.2 takes place on autoclaving the medium used for culture of rye roots and the pH was adjusted aseptically, as described in Methods and Materials 3-6 after the medium had been autoclaved.

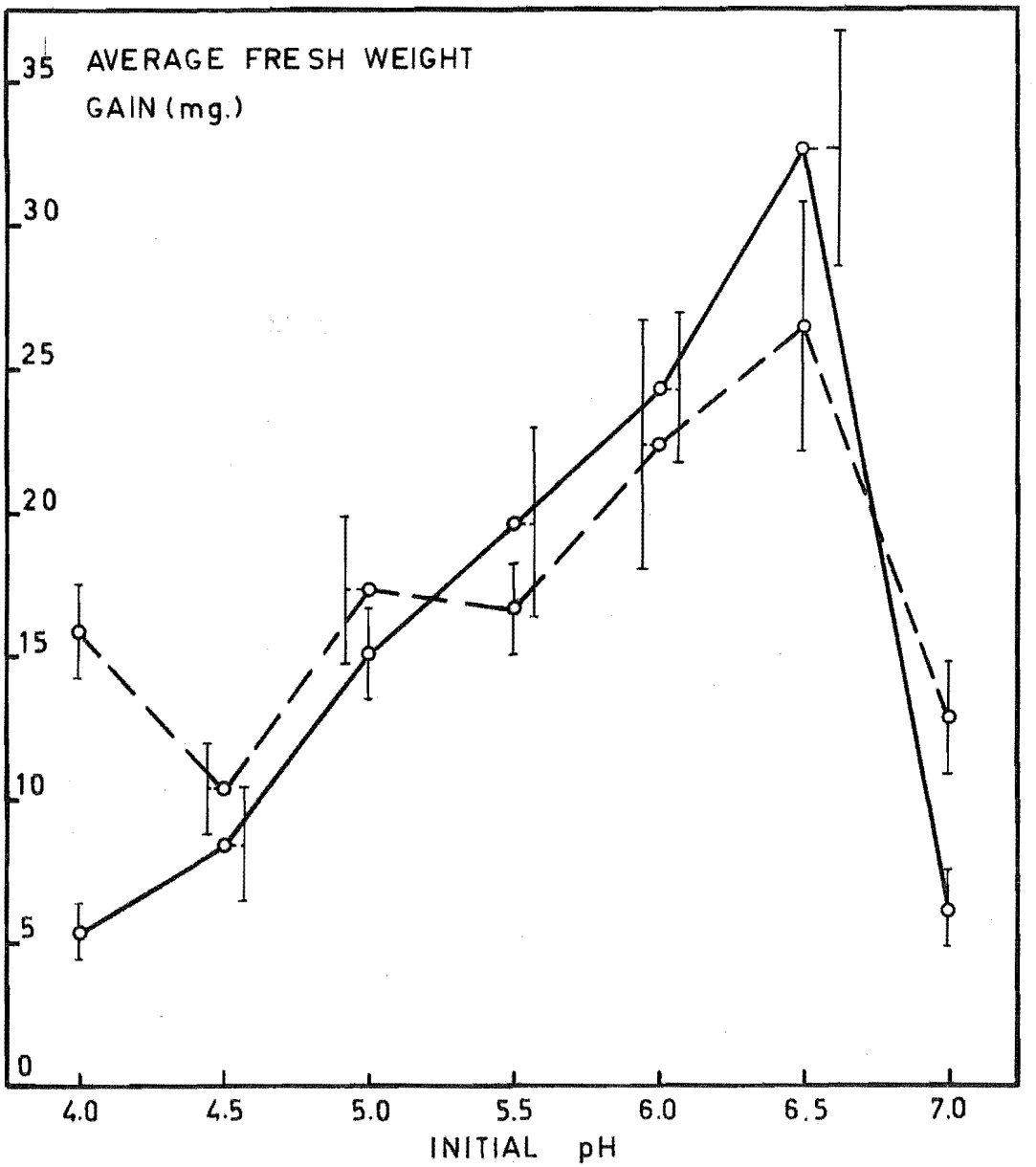
'Tryptophane' medium containing untreated glucose (IV) was prepared as usual and medium containing ammonium nitrate was made by replacing sodium nitrate in this medium with ammonium nitrate at equivalent N concentration. The X5 phosphate medium contained 100 mg./l. KH_2PO_4 instead of 20 mg./l. and the change of potassium concentration was ignored.

'Tryptophane' medium containing untreated glucose	pH 4.0,4.5,5.0,6.0,6.5,7.0.	
with ammonium nitrate in place of sodium nitrate	4.0	6.5
with X5 phosphate	4.0	6.5

'Tryptophane' medium (IV) with untreated glucose	Initial pH value		B Fresh weight gain(mg.)	A Dry weight gain (mg.)	A/B x100	No.	Final pH
	4.5	P1	10.3±1.7	0.9	9	8	4.7
		P2	8.4±2.0	0.6	7	7	4.4
	6.0	P1	22.4±4.3	1.8	8	9	6.1
		P2	24.3±2.6	1.7	7	10	6.0
with ammonium nitrate replacing sodium nitrate	4.5	P1	10.2±1.1	0.8	8	10	4.3
		P2	7.0±1.2	0.5	7	10	4.2
	6.0	P1	19.0±1.5	1.5	8	10	4.3
		P2	22.0±1.9	1.5	7	9	4.1
with times five potassium phosphate (100 mg./l.)	4.5	P1	11.4±2.6	1.1	10	7	5.0
		P2	9.3±2.4	0.6	6	8	4.5
	6.0	P1	27.0±3.1	2.3	9	10	6.0
		P2	22.5±1.3	1.6	7	9	5.9

Table 4-5. The growth of rye roots in 'tryptophane' medium modified as shown in the table.

FIGURE 4—5



THE GROWTH OF RYE ROOTS IN 'TRYPTOPHANE' MEDIUM AT SEVERAL pH VALUES.

Results and Discussion

Figure 4-5 (Appendix Table 5-5) and Table 4-5 show that growth was markedly stimulated as the initial pH rose from 4.0 to 6.5 before falling at pH 7.0. The pH rose slightly during growth in the first passage at the lower initial pH values and fell from pH 7.0 to 6.5: the other pH values in the first passage and all the values in the second passage remained more or less constant. This was in marked contrast to earlier experiments in which the pH rose from 4.3 to 6.5 or so but the growth in those experiments was similar to that at pH 6.5 in this experiment, and so was so much greater than that at the lower pH values. Be that as it may, the roots grew much better at the higher pH values so that the pH drift was not of itself deleterious for growth.

The increase of the phosphate concentration seemed to have no effect upon either growth or the change of pH, whereas replacing sodium nitrate with ammonium nitrate brought about a fall of pH to around 4.2 with perhaps a slight reduction of growth of roots at an initial pH of 6.5. At pH vales above 6.0 a marked white precipitate formed during growth and this might have either altered the balance of nutrients or removed a large proportion of an over-abundant nutrient.

That the roots grew poorly at the lower pH values suggested that the vigour of the clonal roots had previously declined, so it was decided to adjust the pH of media after autoclaving to 6.5 and therefore ensure that a rapid growth rate was maintained.

Experiment 6

The utilization of thiamine by rye roots

Thiamine is a sulphur containing vitamin essential for the growth of roots of many plant species (Bonner, 1940), but the requirement of rye roots for this supplement, was not established by Roberts and Street. Although the quantity of sulphur it supplied (0.05 µg./50 ml. medium) contributed little to the sulphur needs of the roots, the requirement of rye roots for it was determined in case it could be left out.

Experimental details

To 'tryptophane' medium (IV) containing Street's vitamins without thiamine, was added thiamine to give the concentrations shown below. In case sufficient of the vitamin was carried over by the tips, two of the treatments were carried on for a second passage. The pH of the treatments was adjusted to 6.5.

'Tryptophane' medium (IV) with thiamine at the concentrations

First Passage (P): 0, 5, 10, 20, 30. µg./l.

Second P: -, -, 10, -, -.

- (10 is the standard concentration)

Results and Discussion

'Tryptophane' medium with Street's vitamins minus thiamine

Concentration of thiamine (µ g./l.)	Passage number	Fresh weight gain (mg.)	No. harvested
0	1	11.8 ± 3.4	6
	2	0.7 ± 0.4	7
5	1	18.6 ± 2.7	9
10	1	13.0 ± 2.3	9
	2	4.8 ± 2.0	8
20	1	13.6 ± 3.1	9
30	1	15.1 ± 3.1	8

From the results in the Table (Appendix Table 5-6) growth is seen to be unaffected by the level of thiamine in the first passage, but in the second the roots scarcely grew in the minus thiamine treatment. Although all the roots grew poorly in the first passage and very poorly in the second, these results suggested that thiamine was an essential vitamin for rye roots and that sufficient was present in a tip grown in plus thiamine medium to allow growth for one passage.

Experiment 7

An investigation of the poor growth of tips in experimental media

The rye root tips grew very poorly during experiments after the clone had been moved to the Ilam site, whereas the clone, although not as vigorous as it had been, still seemed to grow quite well. The same medium had been used throughout for maintenance of the clone, but several changes had been made to the medium used for experiments, so it appeared that these changes, although found to be stimulatory during one or two passages, had become deleterious over a long period; growth in these various media was compared again with that in original clone medium.

The changing of the pH was checked by comparing growth with two initial pH values below 6.5 with growth with an initial pH of 6.5 in 'tryptophane' medium (IV) containing ion exchanged glucose. Growth in the ion exchanged glucose treatment at pH 6.5 was compared with that with untreated glucose in 'tryptophane' medium, both at 6.5; and to check the replacement of yeast extract by tryptophane, growth in the last treatment was compared with a treatment of 'tryptophane' medium containing yeast extract instead of tryptophane.

Any possible deleterious effect of adding sodium and chloride ions and decreasing the nitrate concentration, in the presence of yeast extract, was determined by comparing the yeast extract treatment of 'tryptophane' medium with the same concentration of yeast extract in the 'rye' clone medium (III). The clone was cultured in the latter medium, so if it was the changing of the medium's constituents that had reduced the growth rate, the last mentioned treatment should have the highest growth rate.

Design

Tryptophane medium (IV)

- Ion exchanged 2% glucose
  plus 0.54 ppm tryptophane
  at pH 5.5, 6.0, 6.5.
- Untreated glucose
  at pH 6.5.

'Tryptophane' medium (IV)

	pH value	Tryptophane (0.54ppm)	Yeast extract	Average Fresh weight gain (mg.)	No. harvested
- with ion exchanged glucose	5.5	+	-	4.4 ± 1.1	9
	6.0	+	-	7.1 ± 1.1	9
	6.5	+	-	4.8 ± 1.2	8
- untreated glucose	6.5	-	+	3.2 ± 0.4	7
	6.5	+	-	died	-
'rye clone' medium (III)					
- untreated glucose	6.5	-	+	5.8 ± 1.8	9

Table 4-7 The growth of rye roots in several media.

either plus 0.54 ppm tryptophane
or plus 60 ppm yeast extract.

Rye clone medium (III)

- Untreated 2% glucose
at pH 6.5
plus 60 ppm yeast extract.

Results and Discussion

The fresh weight gains are set out in Table 4-7 (Appendix Table 5-7). The treatment containing tryptophane and untreated glucose failed to grow since the tryptophane was inadvertently omitted, and is therefore not included in the Table. The 'rye clone' medium allowed a similar rate of growth to that found in pH 6.5 'tryptophane' medium, so it seemed that the poor growth in this and earlier experiments could not be ascribed to the changed constituents of the medium. Growth of the roots was better, although not significantly so, at pH 6.0 than at pH 6.5, in contradiction to the results of Experiment 5 so these two pH values were compared again in the next part of this experiment. At the same time the possibility was tested that growth in the 'tryptophane' medium containing untreated glucose and yeast extract was less than the others.

Since the variables of this last treatment gave reasonable growth in at least one of the other treatments, it seemed that no individual variable was inhibitory. The combination of untreated glucose and the inorganic portion of the 'tryptophane' medium, as well as the yeast extract and the same inorganic medium are only found in this treatment. The first combination had been found in the past to be satisfactory for growth so it seemed possible that the combination of yeast extract and the 'tryptophane' medium which had a higher salt content than the 'rye clone' medium, might be deleterious especially as yeast extract itself contains salts and amino acids.

(b) Investigation of pH and yeast extract concentration

In the first part of this experiment, at pH of 6.0 seemed better than pH 6.5 so these two were compared again in media containing untreated glucose, since the ion exchanging should not have had any affect upon the result. The treatment containing yeast extract in the 'tryptophane medium' was repeated to see whether the poor growth was reproducible, and in case the yeast extract concentration influenced the growth rate, two lower concentrations of yeast extract were included.

Experimental details

To 'tryptophane' medium (IV) containing untreated glucose, was added either yeast extract or tryptophane to give the concentrations shown below. The pH of the yeast extract containing medium was adjusted to 6.5 and that of the tryptophane containing treatment is shown below.

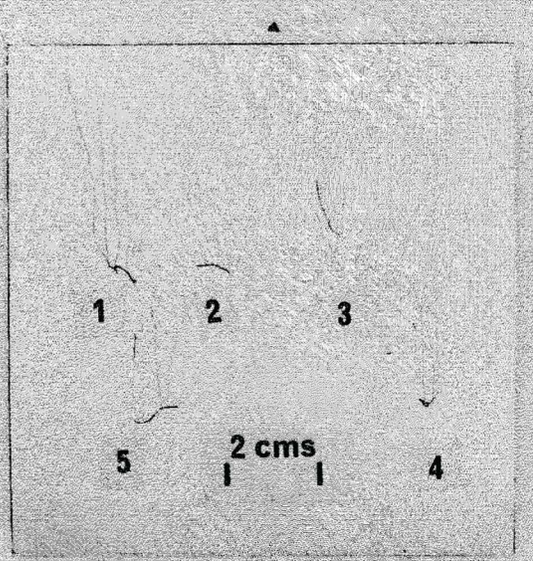
Tryptophane medium (IV)
plus 15, 30, 60 ppm yeast extract at pH 6.5
plus 0.54 ppm tryptophane,
at pH 6.0, 6.5.

Results and Discussion

'Tryptophane medium (IV) with untreated glucose	pH value	Average Fresh weight gain (mg.)	No.
- with either Yeast extract (ppm)			
15	6.5	6.2±1.2	9
30	6.5	6.4±1.1	10
60	6.5	8.0±2.8	9
- or with Tryptophane			
(0.54 ppm)	6.0	6.9±1.9	10
	6.5	8.4±2.3	9

The fresh weight gains were all uniformly poor, as shown in the Table, (Appendix Table 5-7), so no constituent or pH value of the medium which was different from that in the 'rye clone' medium used for the growth of the clone, affected the growth of the rye roots.

FIGURE 4—7 ii



SECTORS—3 WEEKS AFTER BEING SUBCULTURED FROM SEEDLING TIPS OF RYE ROOTS.

The change from excised roots of rye to excised roots of tomato

Shortly after Experiment 7 was completed, the clone also lost vigour and attempts to re-establish it from seeds failed. There seemed little hope of finding in a short time what was causing the rye roots to run down so excised tomato roots were established in culture.

For the culture of these the 'rye clone' medium (III) with the yeast extract omitted and the glucose replaced with 1.5% sucrose - the 'tomato clone' medium (V) was used since this was the medium with which the tomato roots had been cultured.

At first, the roots died and a check of the medium revealed that through a miscalculation, about five times too much zinc had been added to the 'rye clone' medium and to the medium for Experiments 6 and 7 as well as to this lot of 'tomato clone' medium. When the zinc concentration was lowered the tomato roots grew rapidly but another attempt to establish rye roots failed. Figure 4-7ii shows some of the sectors, three weeks old, cut from seedling tips grown for 18 days. The primary laterals were long and lacked secondary laterals.

The culture of tomato roots was therefore continued and subsequent culture experiments were carried out with tomato roots instead of rye, since it was more important to establish which compounds were available to any one plant than to one plant in particular, such as rye.

To maintain the vigour of the tomato roots, they were alternately subcultured as sectors or 10 mm. tips. The latter after seven days growth had a main axis with primary laterals in herringbone array. The axis was cut into pieces (sectors) each with four to five primary laterals. After seven days growth the sectors had the tips excised from the primary laterals for the inoculation of experiments or for the maintenance of the clone.

Experiment 8

The reduction of the volume of medium in which one tomato root grew

A considerable sulphur impurity was still present in the medium of Experiment 4 after the glucose was ion exchanged, but this could be further reduced if the roots grew equally well in a smaller quantity of nutrient solution. The quantity of salts and sugar supplied to one root in 50 ml. of medium was far in excess of that needed, but the concentrations of sucrose and nitrate, at least, were optimal (Mwaulaka, 1967), so that it was only possible to reduce the volume and not the concentration.

Reduction of the volume below 25 ml. would have brought about too large an experimental error, since about one ml., which was 4 per cent, evaporated during the autoclaving regardless of the volume of medium. Therefore the growth of roots in this volume was compared with that in 50 ml..

The inorganic components of the 'tryptophane' medium (IV) which has a lower N content than Bonner's were continued since sodium nitrate probably contributed the most to the sulphur impurity, (Table 4-3).

Experimental details

'Low N' medium (VI) was prepared and dispensed into 25 ml. and 50 ml. portions.

Results and Discussion

'Low N' medium (VI)

	B Fresh weight gain (mg.)	A Dry Weight gain (mg.)	100 A / B	No. harvested
50 ml. to a flask	24.5±3.2	1.98	8.1	8
25 ml. to a flask	23.4±1.3	2.05	8.7	9

The fresh weight gains of both treatments shown in the above table were similar so the roots grew equally well in either 25 or 50 ml. of medium. A fresh weight gain of about 25 mg. was typical for a 7 day growth period (Mwauluka, 1967) and so the growth rate of these roots was satisfactory in

25 ml. of medium which was used later in culture experiments with tomato roots.

The satisfactory and usual growth rate justified the continuance of the lower nitrate concentration used for rye roots since any slight increase of growth would have been more than balanced by a probable increase in the sulphate impurity.

Experiment 9

The growth of tomato roots with limiting sulphate concentrations

Since sulphate was the sulphur source available to all the higher plants that have been studied, the growth response of tomato roots to it was established to compare this with their response to other sulphur compounds. Whether sucrose was effectively purified by ion exchange was established at the same time.

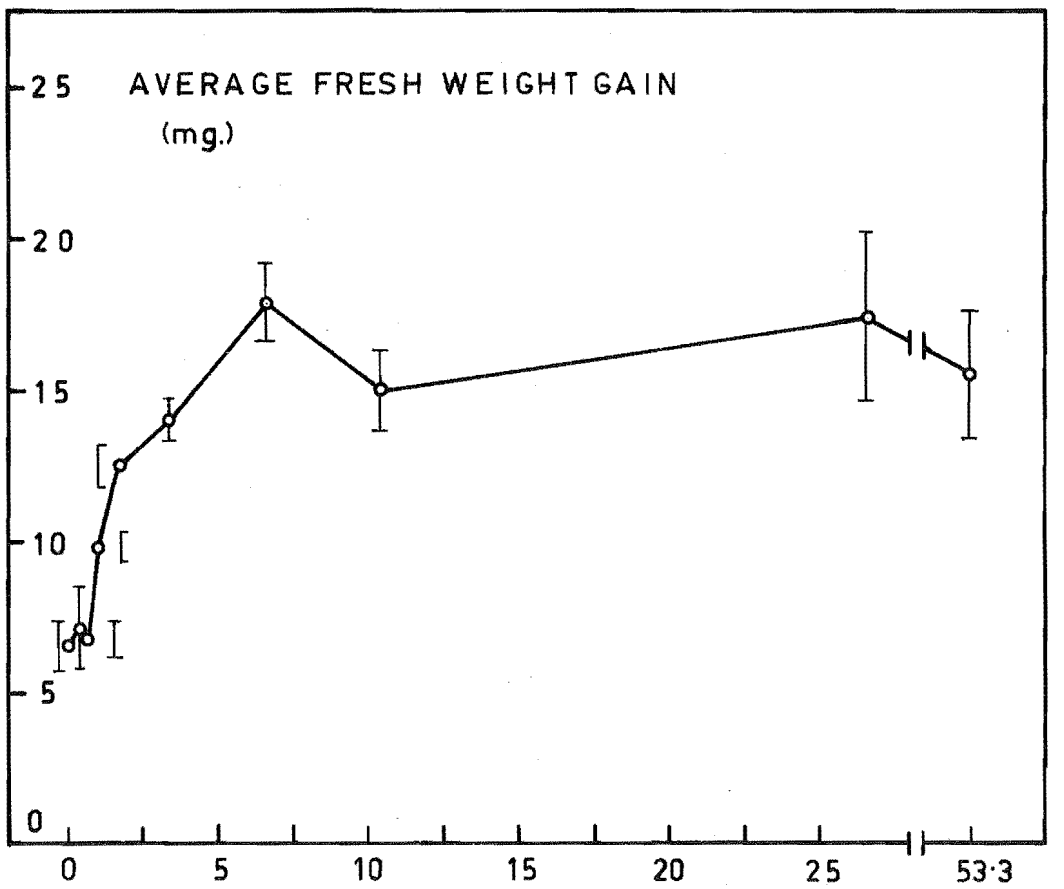
To study the assimilation of sulphur compounds, the choice of a concentration range within and just above those limiting growth made assimilation easier to establish since if a compound was utilized the growth response at limiting concentrations should be approximately linear. Concentrations above those needed for optimum growth are also more likely to be toxic.

Harris (1956) found that amino acids supplied singly to oat embryos frequently inhibited their growth, although the addition of another amino acid sometimes reversed the inhibition. Street, Hughes and Lewis (1960) found that methionine at 1 mg./l. was toxic to tomato roots. Therefore at concentrations above those needed, changes of growth response may not be related to nutrition alone.

From the range of sulphur contents summarized by Gilbert (1951) the quantity of sulphur required for a tomato root to increase 2.5 mg. in dry weight would be about 5 µg. On the same basis the root tip supplied between 0.2 and 0.4 µg. S and from the manufacturers' limits shown in Table 4-3 the medium supplied about 0.4 µg. S - assuming all the sulphur was removed from the sugar.

The smallest quantity of sulphate-S (0.33 µg.) was added to the medium to establish whether the roots would respond to such a small addition. If they did and the growth was twice that in sulphate-omitted medium the suggestion would be that the sulphur impurity was about this size.

FIGURE 4—9



THE GROWTH OF TOMATO ROOTS IN 'LOW N' MEDIUM WITH SULPHATE.

Experimental details

To 'low N' medium (VI) minus sodium sulphate was added sodium sulphate to give the concentrations shown below. The pH of the media after autoclaving was 4.5

'Low N' medium (VI) minus sodium sulphate
containing ion exchanged sucrose
plus 0, 0.33, 0.66, 1.0, 1.66, 3.33, 6.66, 10.3,
26.7, 53.3 µg. sulphate-S/25 ml.

Results and Discussion

The fresh weight gains shown in Figure 4-9 (Appendix Table 5-12) did not increase significantly above the minus sulphate control until 1 µg. of sulphate-S/25 ml. had been added. From this point growth increased approximately in proportion to the quantity of sulphate added until 6.6 µg. S had been added. No significant growth change was then noted up to 55.3 µg. sulphate-S/25 ml. (which was 0.46 times the sulphate in Bonner's medium).

The quantity of sulphur required for optimum growth lay between 3.3 and 6.6 µg.S/25ml., which was slightly more than that expected for an increase between 1.1 and 1.4 mg. dry weight, without taking into account that present as an impurity. This could equally well have meant that the sulphur content of sulphur sufficient tomato roots was higher than expected, but the lag in response to the added sulphate and the poorer growth of the sulphur sufficient roots compared with Experiment 8 suggested rather that the efficiency of yield was not very high.

From this and from the fact that about 8 per cent of the tips aborted, it was concluded that this experiment should be repeated and at the same time further points in the curve below 1 µg.S/25 ml. and between 3.3 and 6.6 µg.S/25 ml. were included.

Before this was done the response of tomato roots to a variety of sulphur compounds was examined to be sure that at least some of these were available for growth.

Table 4-9

cystathionine	Escherichia coli	(Lampen, Roepke and Jones 1947)
cystine	Escherichia coli	(Roberts et al., 1955)
methionine	Penecillium notatum	(Hockenhull, 1948)
cysteic acid	Aspergillus nidulans	(Shepherd, 1956)
taurine	Penecillium notatum	(Hockenhull, 1948)
β-sulphonyl propionic acid	Penecillium notatum	(Hockenhull, 1948)
β-sulphonyl lactic acid	Penecillium notatum	(Hockenhull, 1948)
homocystine	Aspergillus nidulans	(Hockenhull, 1949)
choline sulphate	Penecillium notatum	(Hockenhull, 1948)
S-methylcysteine	Neurospora crassa	(Ragland and Liverman, 1956)
cyst(e)amine	Penecillium notatum	(Hockenhull, 1948)

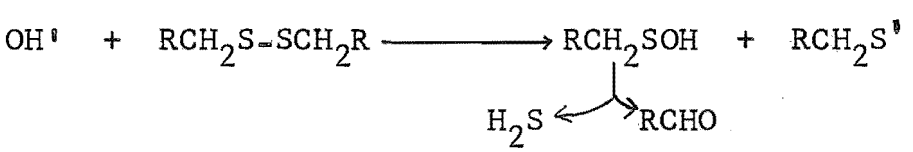
The Choice of sulphur compounds for nutritional studies

From the literature, the compounds which are sufficiently stable to remain relatively unchanged in the medium for 7 days and which have supplied the sulphur requirements of some microorganism are shown in Table 4-9. Postgate (1963) summarized data for the stability of sulphur compounds and since sulphite, sulphide and thiosulphate are all unstable, (the latter at pH values below 5) they were exluded, although these compounds are postulated as intermediates of sulphate reduction.

Methionine and S-methylcysteine form sulphoxides by air oxidation. The rate of oxidation in nutrient solutions was unknown, but Thompson et al. (1959) recorded that methionine oxidation is catalyzed by strong acid. Even though some sulphoxide was formed it was possible that the roots would reduce it to methionine. In the nutrition of rats, Bennett (1939) found that methionine sulphoxide was equivalent to methionine so that these animals reduced methionine sulphoxide.

Thiol compounds such as cysteine and cysteamine in solution are readily oxidized by air. Metal ions such as iron or copper catalyze the reaction. Since these elements were present in the nutrient solution along with EDTA which reduces the rate of oxidation by chelating metal ions, the rate of disappearance of thiols could not be estimated with any accuracy. However Crawhall and Segal (1966) reported that a 4 mM cysteine solution at pH 7.4 was almost oxidized after 20 min and with 10 mM EDTA present 30% remained after 90 min. so that all of the thiol could be expected to disappear before the beginning of the culture period, which was about four days after preparation of the medium.

Rosenthal (1955) agreed that disulphides such as cystine are subject to hydrolytic cleavage especially under alkaline pH conditions



and he suggested that this cleavage of disulphides produced an ultra-violet absorbing chromophore. Below a critical pH the chromophore was not formed and for cystine this pH was 4.5. This suggests that cystine too might be unstable in solution at pH values of about 6.5, but Kolthoff, Stricks and Kapoor (1955) found no measurable decomposition of cystine at pH 7.7 after 21 days incubation in air free buffer. Since the reaction does not involve air oxidation the presence of air in nutrient solutions can be ignored. Hence cystine was concluded to be stable in the nutrient solutions as their pH was less than 7. The stability of cystathionine was unknown although from the synthesis described by Armstrong (1951) it was stable to either ammonia or acid.

From the stable compounds, those more directly implicated in normal sulphur metabolism were investigated first, especially those related to the synthesis of cystine and methionine.

Experiment 10

An exploratory investigation of the utilization by tomato roots of several organic sulphur compounds

Whether tomato roots would grow with sulphur sources other than sulphate was initially investigated by testing the utilization of elemental sulphur, cystine, cysteine, taurine, cysteic acid and methionine for only one growth passage and with a limited range of five concentrations of each source.

The results which are shown in Appendix Tables 5-9, 5-10, and 5-11 were inconclusive because a considerable sulphur impurity was present, which fluctuated to give at times growth equal to that in optimum sulphate concentrations. Also since the average fresh weight gain in optimum sulphate concentrations was frequently below 20 mg., the roots grew so poorly that their growth response to the sulphur compounds was less than it would otherwise have been.

However, responses which were suggestive of utilization for growth were found for cystine, cysteic acid, cysteine (i.e. added cysteine) and elemental sulphur. Methionine was inhibitory at concentrations above 4 µg.S/25 ml..

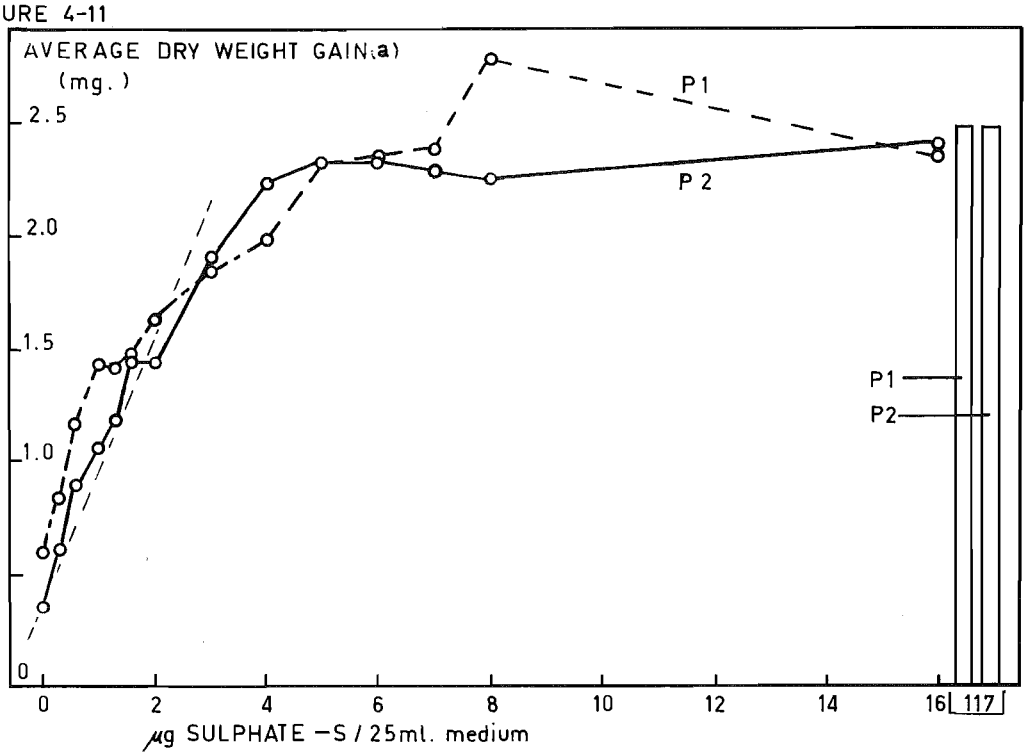
These responses were further investigated since their interpretation was so difficult.

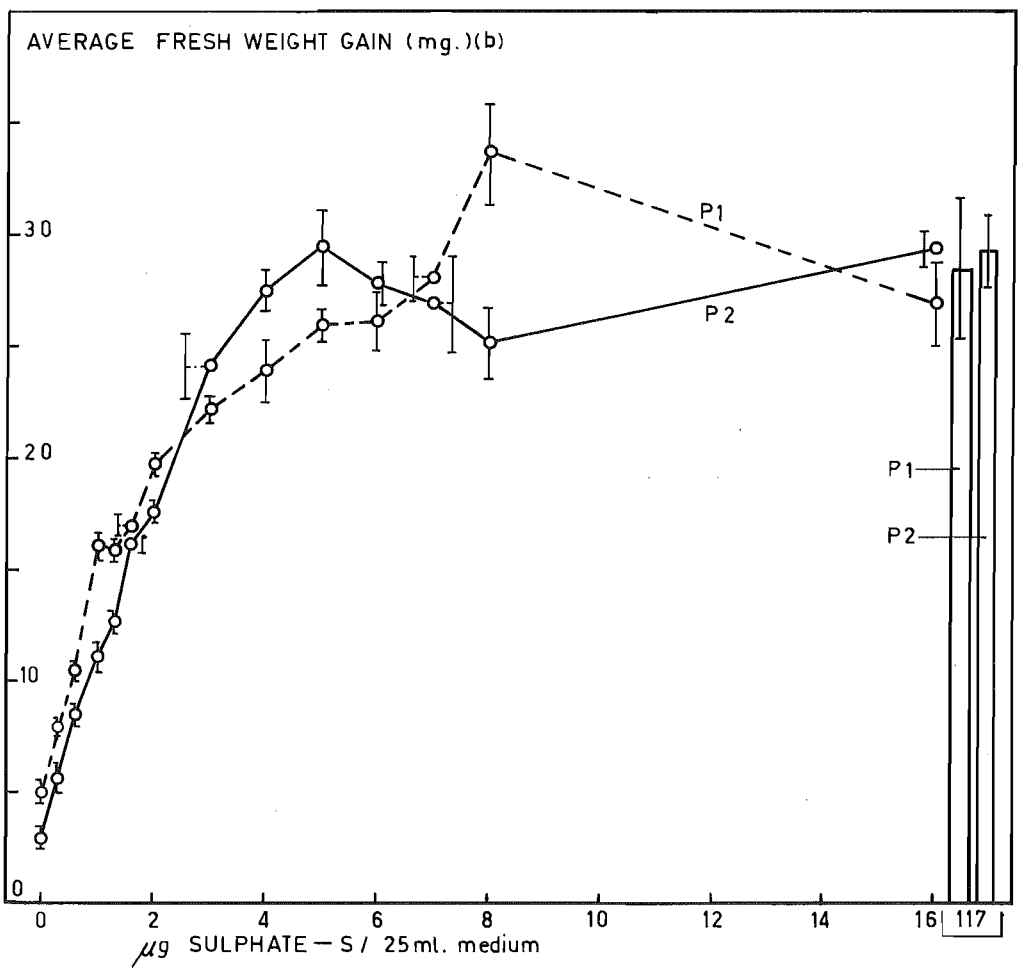
In later experiments the roots were grown through two passages to exhaust the reserves of sulphur present in the inoculum. The vigour of the tomato clone had declined during this experiment and since Mwauluka (1967) had shown that increasing the magnesium concentration 20 times increased the growth of tomato roots the medium was modified to contain more magnesium.

The nitrate concentration of the 'Low N' medium was raised at the same time to that of Bonner's medium, in case it limited growth, since the increased growth in the higher magnesium concentration which was now probably the largest source of sulphur impurity, would have more than compensated for any increase of sulphur impurity from an increase of nitrate concentration.

In the course of this experiment it was noted that the pH of media which had remained for a while in contact with the rubber of the dispensing burette, had a much lower pH than the bulk of the medium. It seemed that sulphuric acid from the chromic acid had soaked into the rubber and was released into the medium despite thorough washing with water. This explained the high sulphur impurity in the media, especially the greater impurity in the organic sulphur media since these had been in contact with rubber several times. In subsequent experiments fresh latex rubber tubing, cleaned as described in Methods and Materials 3-6, was used each time.

FIGURE 4-11





THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH SODIUM SULPHATE. HISTOGRAMS-MEDIUM CONTAINED UNTREATED SUCROSE. CURVES-MEDIUM CONTAINED ION EXCHANGED SUCROSE.

Experiment 11

Growth of tomato roots with varying concentrations of sodium sulphate

In Experiment 9 the roots did not respond to additions of sulphate below 1.0 µg.S/25 ml. and maximum growth was reached when between 3.3 and 6.6 µg.S/25 ml. had been added. In this experiment the range of concentrations covered these regions of the curve more fully.

Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged or untreated sucrose was added sodium sulphate as shown below. The pH of the media was 5.0 after autoclaving.

'High magnesium' medium (VIII)

with ion exchanged sucrose

plus sodium sulphate at 0, 0.3, 0.6, 1.0, 1.3, 1.6, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 16 µg.S/25 ml. medium.

with untreated sucrose

plus sodium sulphate at 117 µg.S/25 ml. medium.

Results and Discussion

The tomato roots responded to the smallest addition of sodium sulphate, as shown in Figure 4-11 (b), (Appendix Table 5-12), and the fresh weight gain increased with the quantity of sodium sulphate until 4 to 5 µg.S/25 ml. had been added, when a plateau was reached. In the second passage the fresh weight gain was less than in the first at lower sulphate levels and was greater between 3 and 5 µg.S/25 ml.. For quantities of sulphate greater than the optimum the increase of fresh weight was generally the same for both passages although quite large fluctuations occurred along this plateau.

The dry weight gain, Figure 4-11 (a), followed the increase of fresh weight, although at the lower sulphate levels the dry weight gain was proportionately larger (Table 4-11). The dry weight gains for both passages corresponded more closely along the plateau than the fresh weight gains.

The smaller fresh weight gain in the second passage below the sulphur concentration of 1.3 μg.S/25 ml. was ascribed to a depletion of the sulphur reserves contained in the tip of the first inoculum. This quantity was relatively small compared with that required for optimum growth since the curves of the two passages tended to merge above 3 μg.S/25 ml. and the amount of sulphur required for optimum growth in the second passage was not greater than that in the first passage.

Gilbert (1951) concluded that the sulphur contents of higher plants lay between 0.1 and 1% of the dry weight. Martin and Walker in their 1966 review presented a table showing that the sulphur contents of plants at the critical level lay mostly between 0.2 and 0.3%. The critical level is that when the sulphur content begins to limit growth.

Examining the relation between dry weight yield and sulphur content, Merced (1952) found for lucerne that at the critical level the sulphur content was approximately 0.2% of the dry weight and fell rapidly with decreasing yield, brought about by sulphur deficiency, to around 0.1% at low yield. When the maximum yield was approached, the sulphur content of the lucerne rose steeply above 0.2% to about 0.4%.

From this evidence the dry weight gain of tomato roots was expected to be proportional to the sulphur content and further the sulphur content of deficient roots would rise with the increasing sulphate supply, which would explain the falling off in steepness of the curve as the optimum is approached.

The sulphur content of 0.18-0.22% of the dry weight for tomato roots (see below), at optimum growth with the lowest added sulphur, was consistent with the values which were summarized by Martin and Walker (1966). Dijkshoorn, Lampe and van Burg (1960) concluded that the organic N:S ratio of sulphur and nitrogen sufficient plants was relatively constant, which was supported by Pumphrey and Moore (1965). Dijkshoorn et al. found that the organic sulphur to organic nitrogen ratio of sulphur

sufficient rye grass was 0.27 gram atoms on the average. Mwauluka (1967) found for this tomato clone in the same medium that its residual nitrogen content averaged 3.9% of the dry weight. From the ratio of Dikjshoorn et al. this would give the sulphur content as 2.4% of the dry weight. These values are therefore all consistent with the dry weight yield for added sulphate and suggested that most of the latter was assimilated by the roots.

The lowest amount of sulphur required in the second passage for the optimum fresh weight gain of one root was 4 to 5 µg.S which correspond to 0.18 and 0.22 per cent of the respective dry weight gains. When quantities of sulphate-S between 0 and 2 µg. were added, a straight line drawn through these points (Figure 4-11 (a)) shows that the sulphur added was about 0.17 per cent of the increase of dry weight gain. Since this response and that overall were similar, the dry weight gain was related to the added sulphate and was limited almost entirely by the quantity added. So from the earlier conclusion that the sulphur content of S deficient plant material was a constant or increasing fraction of the dry weight, the sulphate added was taken up until only a small fraction remained in the medium. Since the roots responded to the addition of 0.3 µg. sulphate-S this fraction was, at low sulphate levels, less than 7 per cent of the quantity needed for optimum growth. That is for quantities of sulphate below those needed for maximum growth.

Because the roots assimilated most of the added sulphate even at low quantities, the size of the sulphur impurity in the medium was proportional to the dry weight gain that it produced, unless there was a concentration of sulphate below which the roots did not take up this ion. This was precluded, provided that the impurity was sulphate, by the result for Experiment 22 in which eleven or twelve 7 day old tomato roots, which were estimated to weigh between 20 and 25 mg. on the average, took up 40 per cent of the radioactivity one hour after 50 µC. of carrier-free sulphate had been added. This quantity of (^{35}S) sulphate would have

increased the sulphate quantity by only 3 $x10^{-3}$ µg. so that, ignoring the free space uptake, 40 per cent of the sulphate impurity present in 25 ml. of medium was taken up by these roots. If there was a concentration below which uptake was not possible this was so low that it could for practical purposes be ignored. From the growth response in sulphate-omitted medium, the sulphate impurity of the medium was about 0.5 µg.S for the second passage of this experiment.

The failure to find a theshold for sulphate uptake was also reported by Bohinski and Mallette (1965) for Escherichia coli, the growth of which responded to sulphate concentrations above 10^{-7} M (0.08 µg.S/25 ml.)

Experiment 12
The growth of tomato roots with cysteic acid and taurine

I (a) Growth with cysteic acid

Shepherd (1956) suggested that cysteic acid might be reduced to cysteine by *Aspergillus nidulans*, so it offers a possible pathway of sulphate reduction primarily through organic intermediates.

Cysteic acid can also give rise to taurine in chicks and to sulphate in microorganisms present in the gut of animals (Young and Maw, 1958). Several possible metabolic routes are therefore available to explain its apparent utilization by tomato roots in Experiment 10. Since it seemed to be slightly toxic at 32 µg.S/25 ml., concentrations between 0 and 20 µg.S/25 ml. were examined in this experiment.

Experimental details

To 'high magnesium' medium (VIII), containing ion exchanged sucrose, was added cysteic acid or sodium sulphate as shown below. A further treatment of untreated sucrose with sulphate at the standard concentration was included.

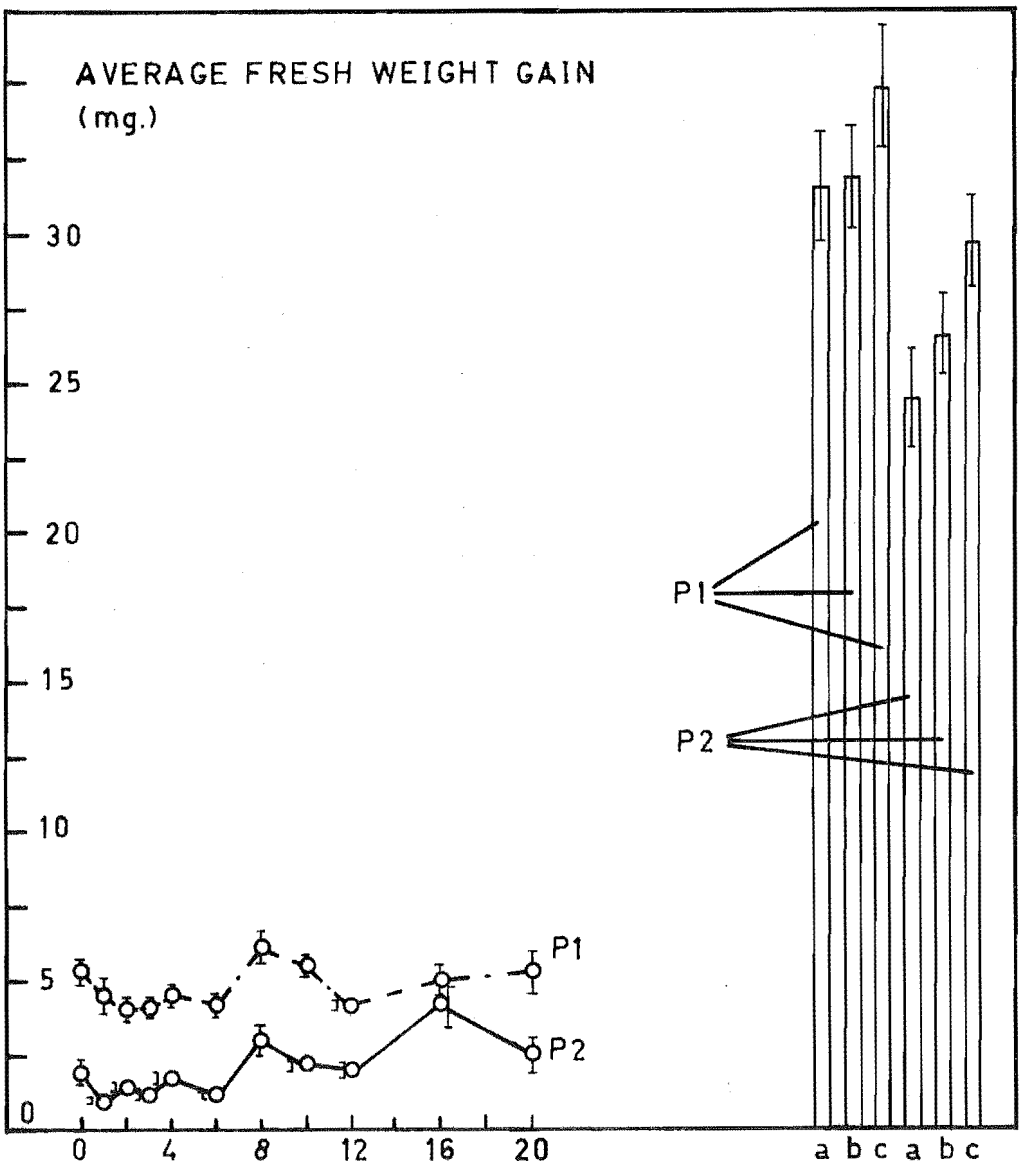
In this and other experiments with sulphur compounds, apart from sulphate, treatments congaining 8 and/or 16 µg. sulphate-S/25 ml. were included to compare maximum growth in the medium containing the sulphur compound under investigation with that with a sulphate concentration found to be greater than optimum in Experiment 11. Two concentrations of sulphate were usually added to improve the precision of the comparison.

'High magnesium' medium

A with ion exchanged sucrose with
either 1,2,3,4,6,8,10,16,20 µg. cysteic acid-S/25 ml.
or 0,8,16 µg. sulphate-S/25 ml.

B with untreated sucrose
plus 117 µg. sulphate-S/25 ml.

FIGURE 4-12 i



μg. CYSTEIC ACID — S/ 25 ml. MEDIUM

GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER CYSTEIC ACID OR SULPATE. SUCROSE ION EXCHANGED EXCEPT FOR HISTOGRAM(c). (a) 8, (b) 16, (c) 117 μg. SULPHATE / 25 ml. MEDIUM

Results and Discussion

Fresh weight gains shown in Figure 4-12i (Appendix Table 5-14) did not increase when cysteic acid was added to the medium. This result was in contrast to that of Experiment 10 in which it was tentatively concluded that cysteic acid stimulated growth.

In Experiment 10 it was concluded that there was quite a large sulphate impurity, so these contradictory results would be reconciled if some comppund which was usually derived from sulphate (and which was not involved in protein synthesis), could also be formed from cysteic acid. If this were the case, cysteic acid would have 'spared' sulphate for protein synthesis, perhaps in a similar way to that found by Davies, Mercer and Goodwin (1966) for Euglena in which cysteic acid was utilized for the synthesis of sulpholipid and did not reduce the uptake of (^{35}S) sulphate into cystine. This possibility was tested in the next part of this experiment.

Experiment 12

I (b) The growth of tomato roots on cysteic acid in the presence of sulphate

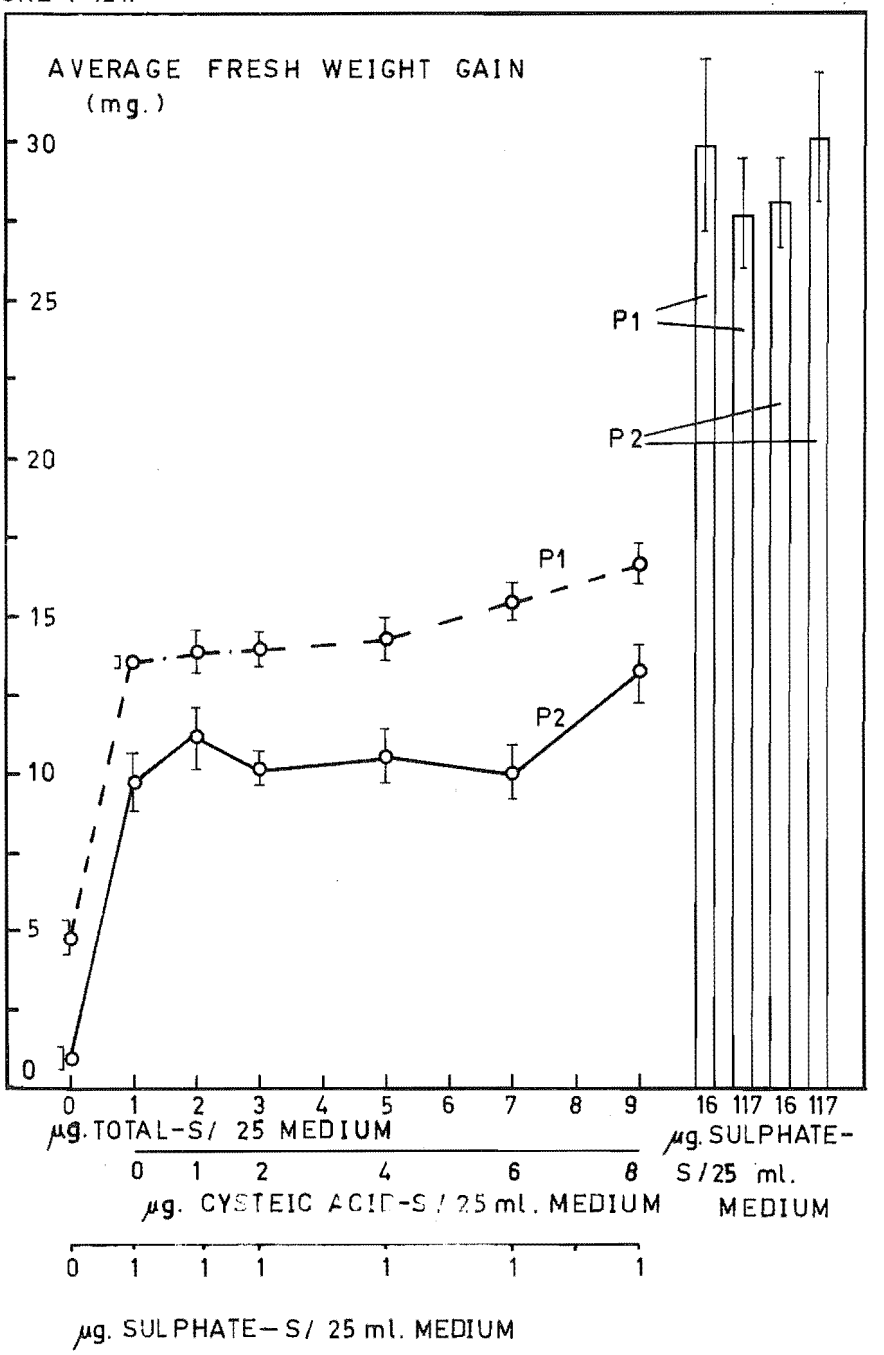
In part I (a) of this experiment cysteic acid did not stimulate the growth of the roots. As a sulphate impurity was present in Experiment 10 in which cysteic acid did stimulate growth, these results could be reconciled if cysteic acid was utilized only in the presence of sulphate. In further support of this contention are the results of Ellis and Davies (1961) who found that glutamic transaminase of cabbage accepted cysteic acid to yield β-sulphonyl pyruvic acid. Cysteic acid did not react as rapidly as glutamate with the enzymic preparation, but these results do at least indicate that cysteic acid may be metabolized by plant tissues.

In this part of the experiment, the possibility was tested by increasing the amount of cysteic acid in a medium containing 1.0 µg. sulphate-S/25ml. This quantity was chosen as it allowed about one half maximum growth and this was approximately the amount of growth in the sulphate-omitted treatment in Experiment 10.

Experimental details

To sulphate-omitted 'high magnesium' medium (VIII) containing ion exchanged sucrose was added 1 µg. sulphate-S /25 ml. sufficient for six treatments. This medium was divided into six aliquots and the quantity of cysteic acid stated below was added to each. This procedure was followed to ensure that equal quantities of sulphate were present in each treatment. A further treatment of the same medium to estimate the sulphur impurity lacked both sulphate and cysteic acid, and another contained only sulphate to provide a standard of comparison. A treatment of untreated sucrose with sodium sulphate was included. The pH of the media was 4.9.

FIGURE 4-12 ii



THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER CYSTEIC ACID AND SULPHATE OR SULPHATE ALONE. SUCROSE WAS ION EXCHANGED EXCEPT FOR THE TREATMENT CONTAINING 117 µg. SULPHATE — S/ 25 ml.

'High magnesium' medium (VIII)

with ion exchanged sucrose

plus µg. sulphate-S /25ml.	and µg. cysteic acid-S /25ml.
0	0
1	0
1	1
1	2
1	4
1	6
1	8
16	0

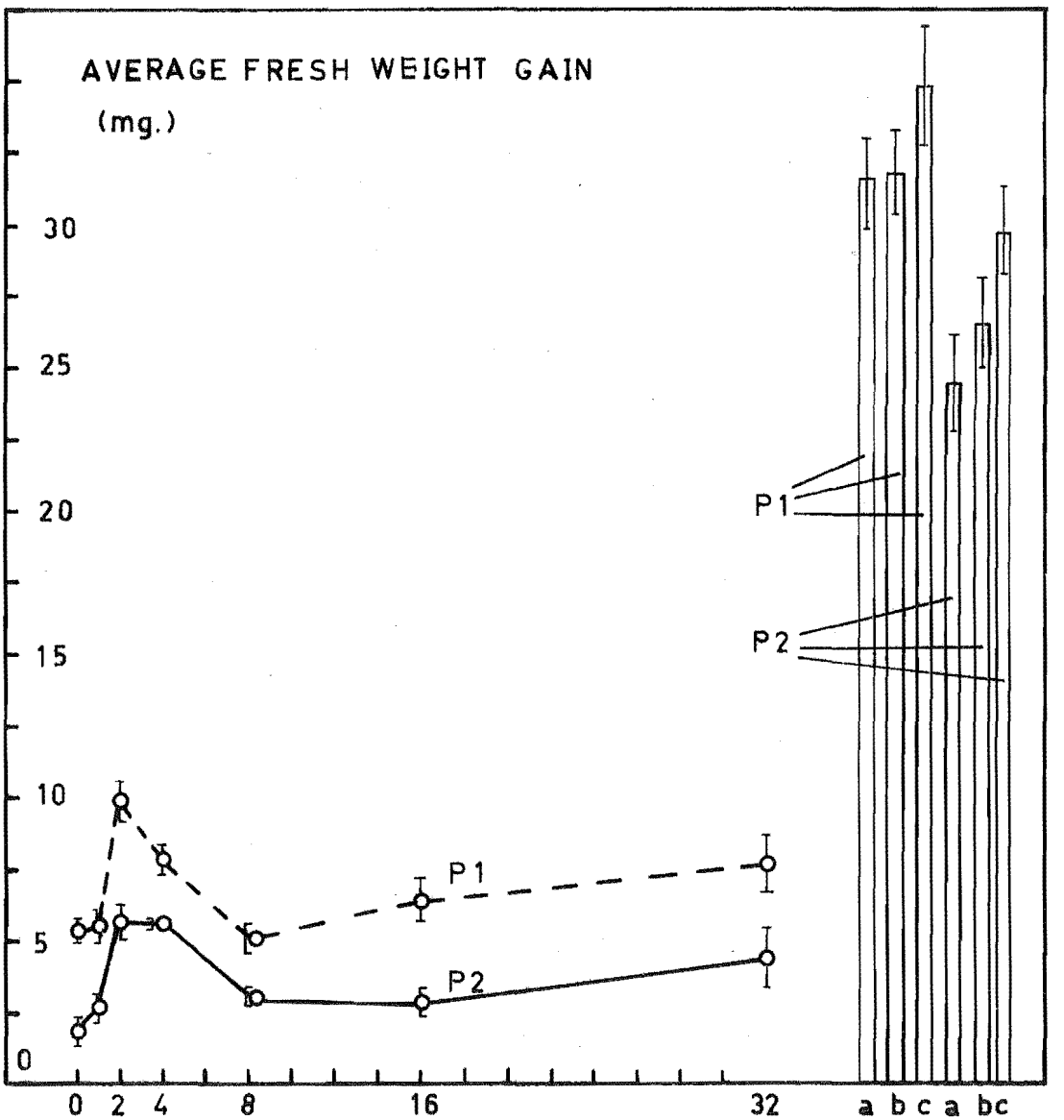
with untreated sucrose
plus 117 µg. sulphate-S/25ml.

Results and Discussion

The fresh weight gains shown in Figure 4-12ii (Appendix Table 5-17) increased slightly at the two highest cysteic acid concentrations. This increase disappeared for the lower cysteic acid concentration - 6 µg. cysteic acid-S/25 ml. - in the second passage. Only the results of one treatment therefore suggested any response to cysteic acid in the second passage and the average fresh weight gain of this did not differ significantly from that of the treatment containing 2 µg. cysteic acid -S/25 ml.. Since cysteic acid at 8 µg.S/25 ml. was greatly in excess of the sulphur requirements of the roots it seemed most unlikely that cysteic acid was assimilated. Certainly it can be concluded that the results of Experiment 10 cannot be explained by a utilization of cysteic acid in conjunction with the sulphate impurity. A possible explanation of these results is that the burette used for dispensing the sterilized cysteic acid solution was contaminated rather more than usual and so the sulphate contamination from this would have been greater for the higher cysteic acid levels, with a corresponding increase of growth.

It was concluded from these results that no evidence could be found for the assimilation of cysteic acid by tomato roots.

FIGURE 4-12 iii



μg. TAURINE — S / 25 ml. MEDIUM

THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER TAURINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR (c). HISTOGRAMS: (a) 8 (b) 16, (c) 117 μg. SULPHATE — S / 25 ml..

Experiment 12

II (a) Growth on taurine

In the hen, taurine is formed from sulphate and was suggested by Martin et al. (1966) to be a precursor of cysteine but it can also be derived from cysteine in other animals (Young and Maw, 1958). Aspergillus niger was reported by Garreau (1941) to oxidize taurine to sulphate, so that if it were used by tomato roots then several pathways of assimilation can be proposed.

Experimental details

As for part I (a) of this experiment and as set out below. The pH of the media was 4.9.

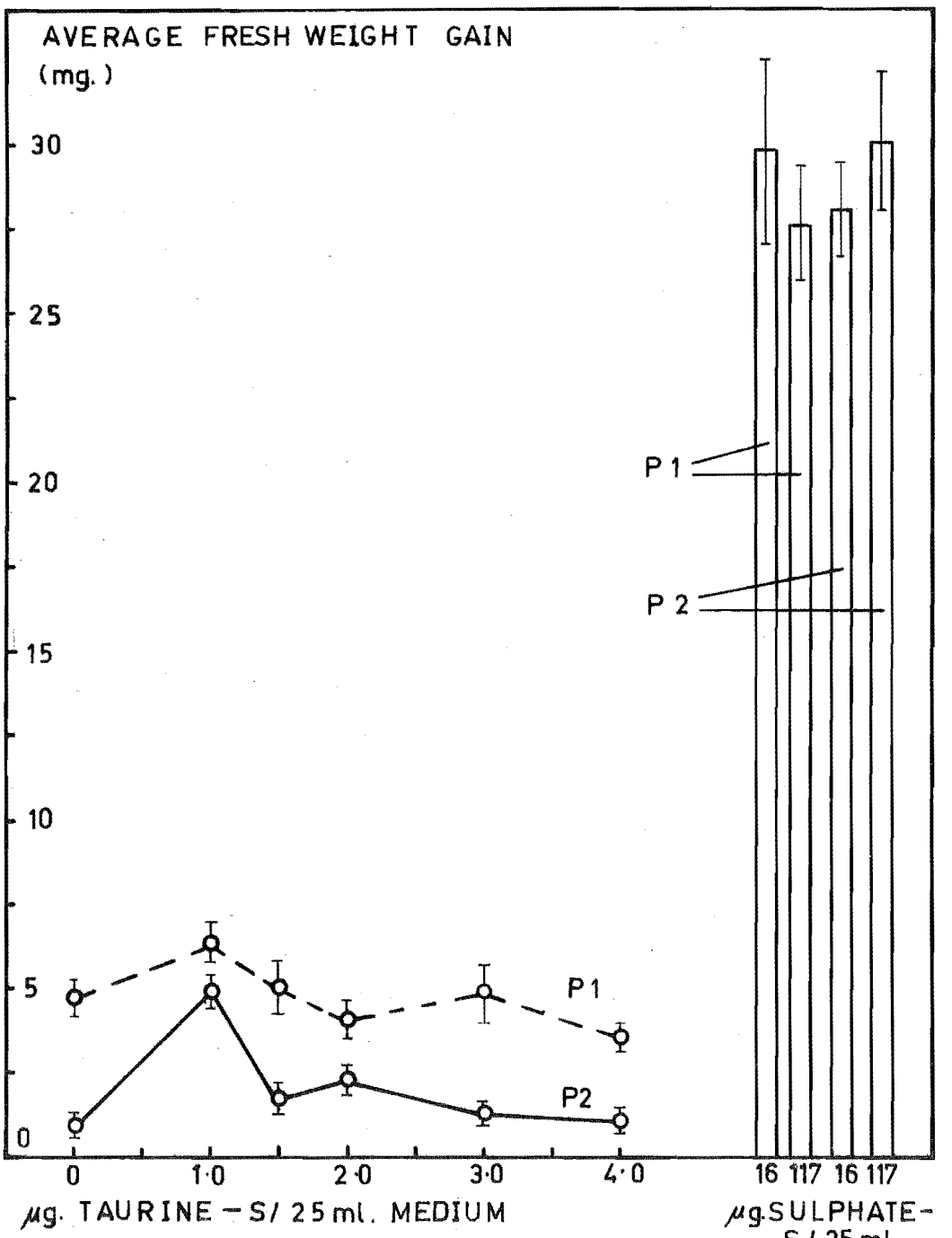
'High magnesium' medium

plus 1, 2, 4, 8, 16, 32 µg. taurine-S/25 ml.

Results and Discussion

The fresh weight gains shown in Figure 4-12iii (Appendix Table 5-14) show a slight stimulation in the treatments with 2 and 4 µg. taurine-S/25 ml.. It could have been concluded from this that taurine was assimilated except that increased growth at higher concentrations would also be expected. A comparison of these results with those of Experiment 10 shows that they were similar in that the curve fell steadily at concentrations above 4 µg.S/25 ml.. This suggested that taurine was inhibitory at the higher concentrations so the region of the curve 0-8 µg.S/25 ml. was examined in more detail for the next part of this experiment.

FIGURE 4–12 iv



THE GROWTH OF TOMATO ROOTS IN HIGH MAGNESIUM MEDIUM WITH EITHER SULPHATE OR TAURINE. SUCROSE WAS ION EXCHANGED EXCEPT FOR THE TREATMENT CONTAINING 117µg. S/ 25ml.

Experiment 12

II (b) The growth of tomato roots on taurine

In Part (a) of this experiment the roots responded to 2 and 4 µg. taurine-S/25 ml. but not to the higher concentrations so suggesting that a small incorporation of taurine at the lower concentrations was inhibited at higher concentrations. This was examined by adding an increased number of treatments in the range 0 to 4 µg.S/25 ml..

Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either taurine or sodium sulphate as stated below. A treatment containing untreated sucrose with sodium sulphate was included. The pH of the media was 4.9.

'High magnesium' medium (VIII)

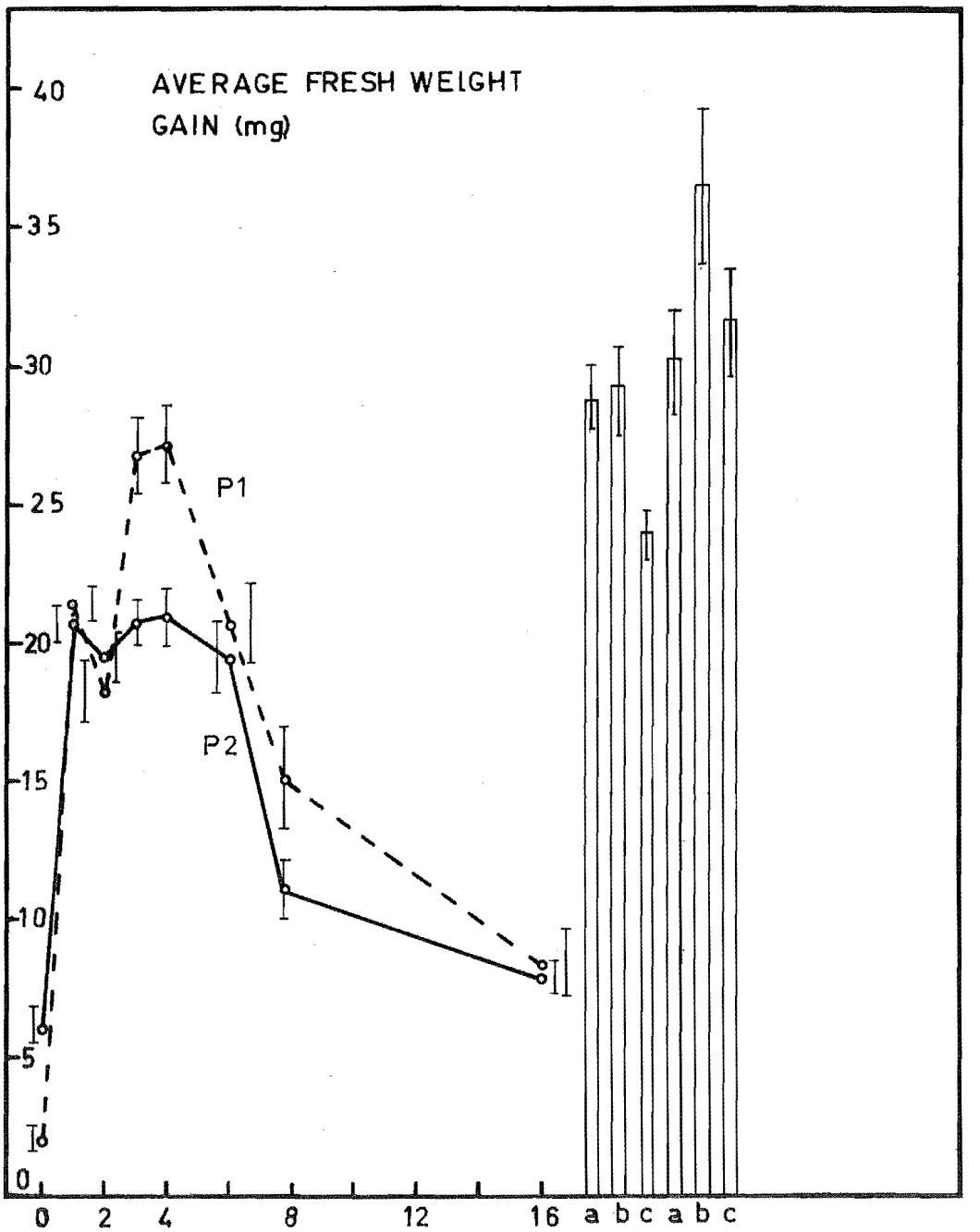
- containing ion exchanged sucrose with
either 1.0, 1.5, 2.0, 3.0, 4.0 µg. taurine-S/25 ml.
or 0, 16 µg. sulphate-S/25 ml.

- containing untreated sucrose
with 117 µg. sulphate-S/25 ml.

Results and Discussion

The fresh weight gains shown in Figure 4-12iv (Appendix Table 5-17) showed that growth of tomato roots was not increased by the addition of taurine in the first passage. In the second passage the 1 µg.taurine-S/25 ml. treatment had a fresh weight gain above those for all the other treatments. This bump in the curve does not parallel that in Part II (a), in which a bump appeared between 2 and 4 µg.S/25 ml.. As occasional fresh weight gains which are greater than the average gain for sulphate-omitted treatments could be expected from the results of Experiment 13, it was concluded that tomato roots were unable to assimilate taurine.

FIGURE 4-13 i



μg. METHIONINE — S / 25 ml. MEDIUM.

THE GROWTH OF TOMATO ROOTS IN HIGH MAGNESIUM MEDIUM WITH EITHER METHIONINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR (c). HISTOGRAMS; (a) 8; (b) 16; (c) 117 μg. SULPHATE — S/25ml.

Experiment 13

(a) The growth of tomato roots with methionine

From the results of Experiment 10, methionine was likely to have been available only at concentrations below 4µg.S/25 ml. since higher concentrations markedly inhibited growth. Although no stimulation of growth was noted in that experiment, its utilization was reinvestigated especially in view of its assimilation by rice and mustard reported by Formin and Astakhova (1959) and since growth of the roots was generally poor in Experiment 10.

Experimental details

To 'high magnesium' medium with ion exchanged sucrose was added methionine or sodium sulphate as stated below. A treatment of untreated sucrose with sodium sulphate was included.

'High magnesium' medium (VIII)
- containing ion exchanged sucrose
either 1, 2, 3, 4, 6, 8, 16 µg. methionine-S/25 ml.
or 0, 8, 16 µg. sulphate-S/25 ml.
- containing untreated sucrose
plus 117 µg. sulphate-S/25 ml.

The pH of the media was 4.9

Results and Discussion

The addition of methionine, in the second passage, to sulphate-omitted medium increased the fresh weight gain of tomato roots up to an optimum at 3 µg.S/25 ml. which was greater than that for the first, as shown in Figure 4-13i (Appendix Table 5-13), and which was similar to that for the sulphate sufficient treatments in the second passage. Amounts of methionine greater than 4 µg.S/25 ml. depressed growth as compared with the optimum.

The fresh weight gain after adding 1 µg.S/25 ml. of methionine was much greater than that in Experiment 11 after adding 1 µg.S/25 ml. of sulphate-S, so this experiment was repeated in case a sulphur impurity was present in the methionine treatments additional to that present in the sulphate containing treatments. Since the minus sulphate

and sulphate containing treatments were prepared without filter sterilization, it was possible that the equipment used for filter sterilization augmented the sulphur impurity in the medium. This possibility was examined and the response to methionine at the lower concentrations reestablished before the results of this part of the experiment are discussed further.

Experiment 13

(b) Confirmation of the results of Part (a)

Methionine appeared, in Part (a) of this experiment, to have been more efficiently used than sulphate for the growth of tomato roots. Nevertheless a greater sulphur impurity might have been present, perhaps introduced by the filter sterilization of the methionine. Filter sterilized portions of double distilled water, equivalent to the amounts added with the methionine solution, were therefore added to the sulphate-omitted medium, so that, if a sulphur impurity was present, the fresh weight gain would increase with the increasing quantities of sterilized water.

To establish whether the greater efficiency was indeed so, that portion of the methionine curve below 2 µg.S/25 ml. was examined in greater detail and compared at the same time with treatments containing 1 and 2 µg. sulphate-S/25 ml.

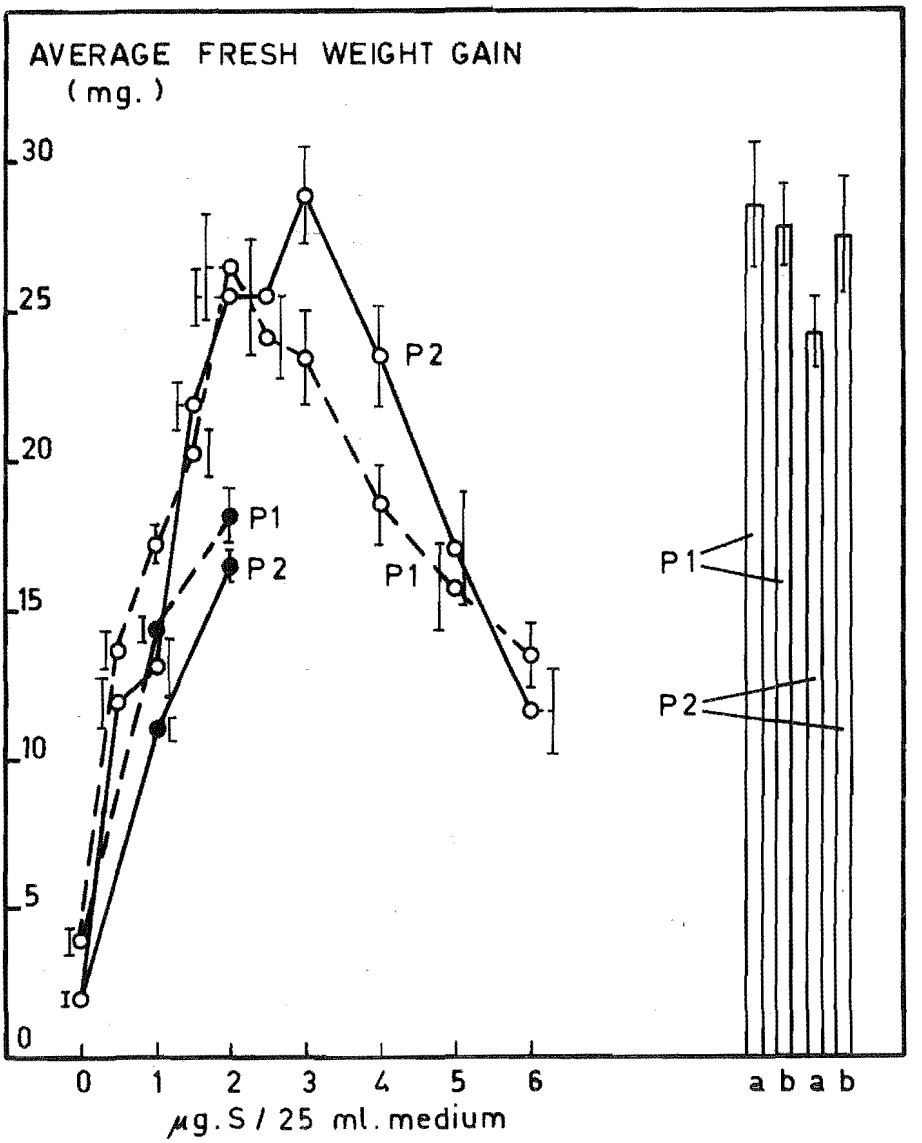
Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either methionine, sodium sulphate or double-distilled water. A treatment containing untreated sucrose and sodium sulphate was included. The pH of the media was 5.0.

'High magnesium' medium (VIII)

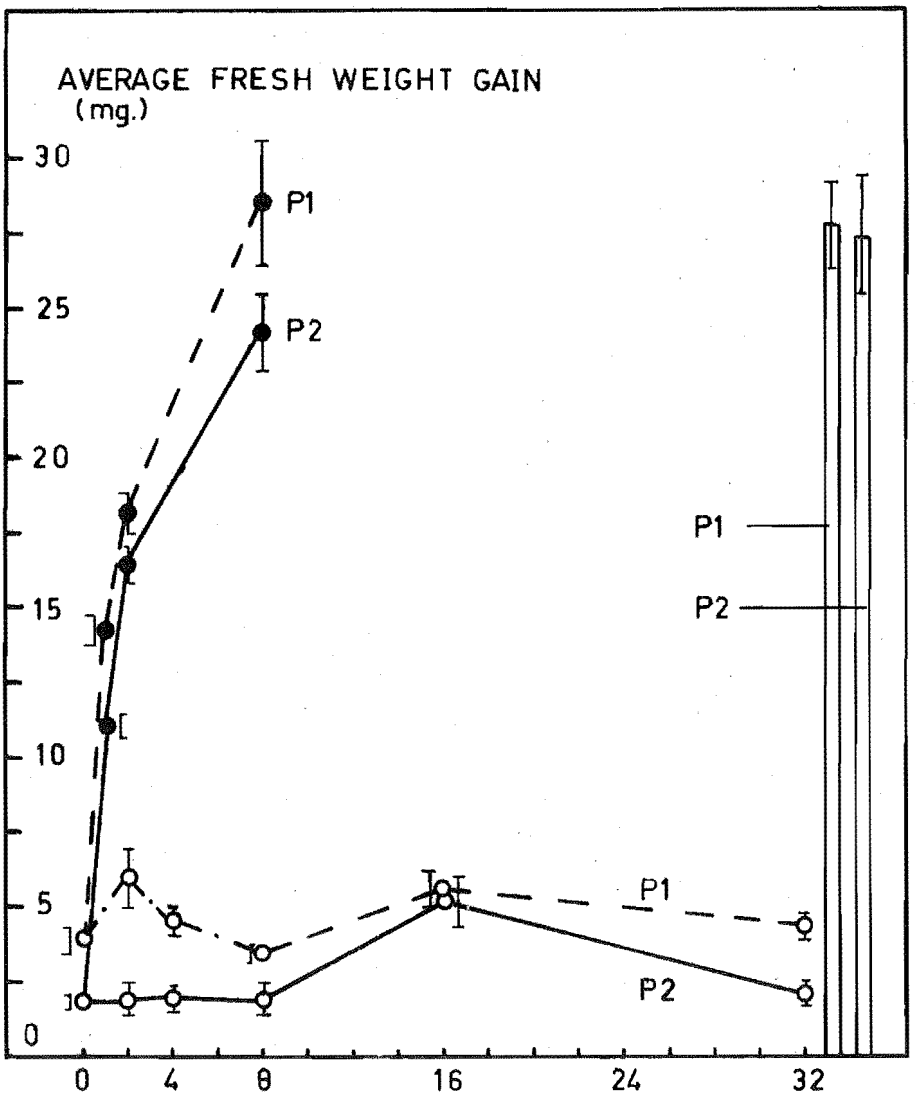
- containing ion exchanged sucrose with
  either methionine 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, µg.S/25 ml.
  or sodium sulphate 0, 1, 2, 8 µg.sulphate-S/25 ml.
  or double distilled water 2, 4, 8, 16, 32 ml./550 ml. medium.
- containing untreated sucrose with 117 µg.sulphate-S/25 ml.

FIGURE 4-13 iii



THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER METHIONINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR TREATMENT b. HISTOGRAMS: a – 8μg. SULPHATE–S / 25ml.; b - 117μg. SULPHATE–S/25ml.. CURVES: o—o, o--o METHIONINE; ●—●, ●--● SULPHATE.

FIGURE 4-13 ii



ml. OF DOUBLE DISTILLED WATER ADDED TO 500 ml. MEDIUM, OR μg. S/25 ml. MEDIUM.

THE GROWTH OF TO TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER DOUBLE DISTILLED WATER OR SODIUM SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR TREATMENT 'a'. HISTOGRAMS: (a) 117 μg. SULPHATE — S/ 25ml.. CURVES: ●—●, ●- -● SULPHATE; ○—○, ○- -○ DOUBLE DISTILLED WATER.

Results and Conclusions

(i) Filter sterilized double distilled water

The fresh weight gains in Figure 4-13ii (Appendix Table 5-15) show that growth of all treatments fluctuated around 5 mg. for the first passage and, in the second, four of the treatments had fresh weight gains of 2 mg. and the fifth 5 mg.. In spite of this one fluctuation the amount of growth was not related to the quantity of water added in either passage. The treatment with greater growth in the second passage could be ascribed to a small sulphate impurity being present in the flask used for preparing the treatment, or to a fluctuation of growth rate. This result suggested that care must be taken in interpreting stimulation of growth in one or two treatments only, as occured in Experiment 12.

However the greater efficiency of utilization of methionine in section (a) of this experiment cannot be ascribed to a greater sulphur impurity in filter-sterilized treatments as compared with sulphate treatments sterilized entirely by autoclaving.

(ii) Methionine

The fresh weight gains shown in Figure 4-13iii (Appendix Table 5-15) were stimulated in both passages slightly more at 1 μg.S/25 ml. and much more at 2 μg.S/25 ml. than by equivalent quantities of sulphate. The dry weight gains shown in Table 4-13 followed the same pattern, as evidenced by the dry weight percent. An optimum was reached at 2-3 μg. methionine-S/25 ml. compared with 4-5 μg. sulphate-S in Experiment 11. In both parts of this experiment, growth at concentrations greater than the optimum was somewhat better in the second passage and this suggested that some adaption of tomato roots to methionine may have taken place.

Although the growth response to methionine was more rapid than that to sulphate, the added sulphur as a per cent of the increase of dry weight yield was only slightly lower for methionine at low yield (0.10 for 0-2 μg.S in the second passage) compared with sulphate (0.13 for 0-1 μg.S

Table 4-13

'High magnesium' medium (VIII)
- containing ion exchanged sucrose

	µg.S /25 ml.	Average dry weight gain (mg.)		Average dry weight gain as per cent of Average fresh weight gain	
		First passage	Second passage	First passage	Second passage
plus methionine	0.5	1.20	1.1	8.8	9.3
	1.0	1.54	1.25	8.9	9.6
	1.5	1.53	1.82	7.6	8.3
	2.0	2.06	2.10	7.8	8.3
plus sulphate	0	0.44	0.22	11.3	12
	1	1.26	0.98	8.8	8.9
	2	1.43	1.33	7.9	8.1
	8	2.14	2.11	7.5	8.7

in the second passage). Both of these are well below the values of 0.2 to 0.3% quoted by Martin and Walker (1966) for sulphur sufficient plants at the critical level and approached the lower limit of 0.1% reported by Merced (unpublished, in Martin and Walker) for sulphur deficient lucerne. The critical level is the sulphur content below which the yield falls.

Since the response to methionine was almost linear up to an optimum at about half the sulphur needed for an optimum in sulphate, the optimum growth response to methionine is characterized by a lower sulphur content on a dry weight or fresh weight basis. For optimum growth at the critical level with methionine, the ratio of added S to the dry weight was 0.1% whereas that for sulphate was between 0.18 and 0.22 in Experiment 11. This quantity of 0.1% was also much less than the 0.24% calculated from the protein S:N ratio of Dijkshoorn et al.(1960) and the insoluble nitrogen content of the roots. The growth response to sulphate and methionine at 2 μg.S/25 ml. in this experiment was consistent with the S content of the sulphate grown roots being much greater than that of the methionine grown roots.

This more efficient growth with methionine can be explained by either the overall protein content falling, or by protein formed changing to types with fewer sulphur amino acids, or else by only some of the sulphate being used for protein synthesis. In their review of the ratio of organic sulphur to organic nitrogen in plants, Dijkshoorn and Van Wijk (1967) presented results which show that since the fall of cytoplasmic protein was proportionately greater than the fall of chloroplast protein, the ratio of protein S to protein N rose in sulphur deficient leaves. Apart from the fact that this increase may not apply in roots, the change is the reverse of that needed to explain better growth in methionine. In lucerne, the proportion of cystine and methionine in the protein remained the same in sulphur deficient plants so such a change does not seem very likely.

Martin and Walker in their 1966 review showd some results of Merced for the sulphate content of lucerne. With increasing sulphate supply the level of sulphate rose in the deficient plants from about 50 ppm to 500 ppm at the critical level and then rose steeply. Since 500 ppm is 0.05% and the total sulphur added was about 0.2% of the critical dry weight of the tomato roots, the proportion of sulphate added which was not used for protein synthesis was insignificant.

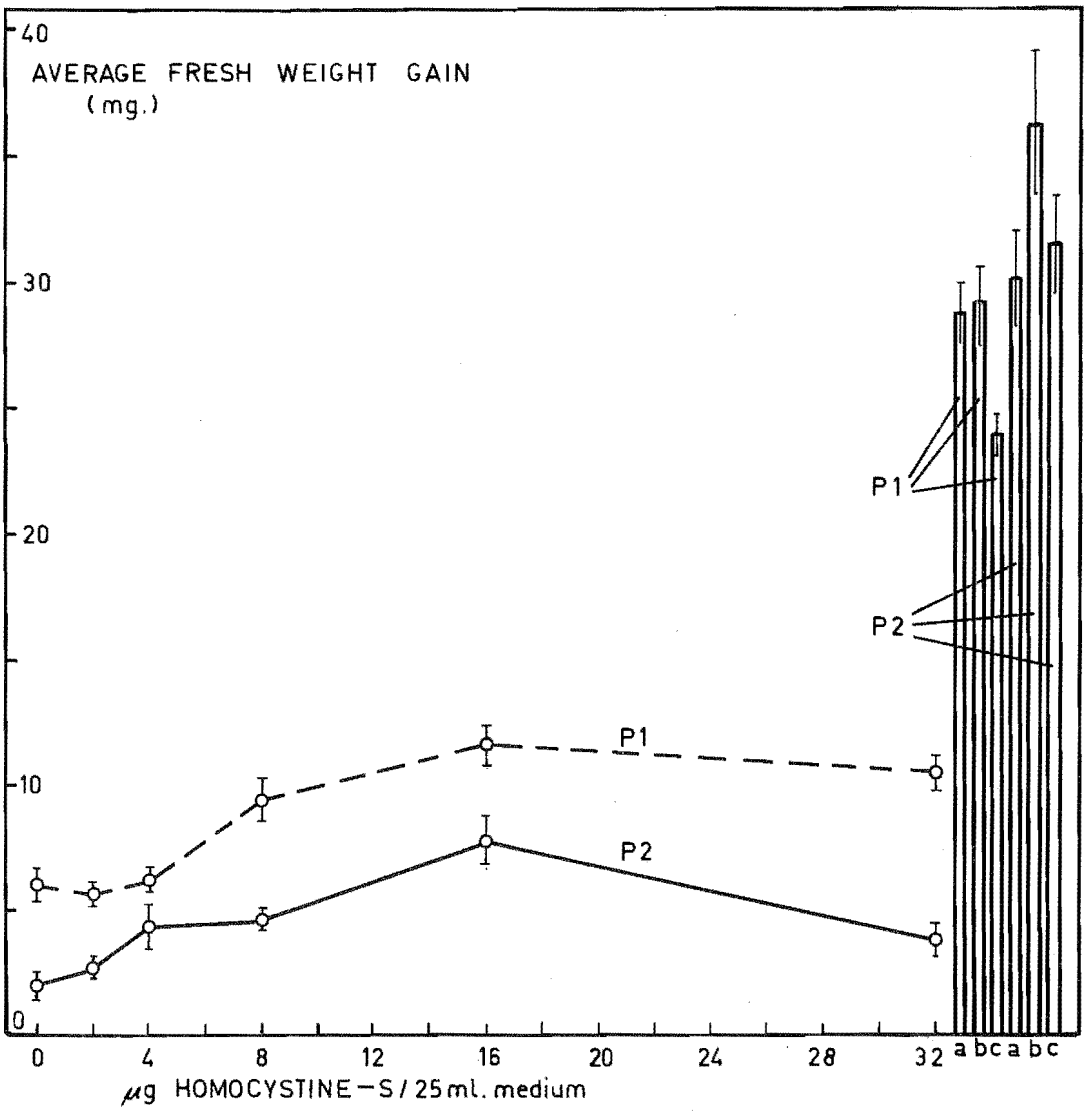
Dougall (1966) reported that the ratio of the α-amino content to the dry weight of Paul's Scarlet Rose tissue rose 20% to a maximum after 4 days of growth and then fell to 20% below the initial ratio after 10 days. The ratio of dry weight to fresh weight reached a maximum at 4 days also. Brown and Broadbent (1950) examined the changes of protein content and dry weight of pea seedling cells with increasing distance from the tip. The protein content N to dry weight ratio fell from 8.5% at the tip to 5.3% at 4.4-4.8 mm. from the apex where the maximum dry weight per average cell was first reached. The protein content of an average cell reached a maximum slightly after the dry weight maximum, at 4.8-5.2 mm. from the apex. The latter changes were related to stages of development as possibly were the results of Dougall but the difference between methionine and sulphate fed roots was not so related.

However, of the alternatives considered above the fall of protein content in relation to the dry weight seems the most likely explanation of the difference between growth with methionine and sulphate. Since the overall growth pattern of the roots as well as the fresh and dry weight gains in optimum sulphate and methionine were similar it was most unlikely that the protein content in the growing cells changed. Therefore the proteins of mature cells only, for instance those of the cortex, would have been hydrolyzed and the sulphur amino acids either oxidized to sulphate or translocated as such to the apices for reutilization. The protein sulphur amino acids of methionine fed roots therefore

turned over more rapidly than did those of sulphate fed roots. This increased rate was not just a response to sulphur deficiency since all of the sulphate supplied was utilized at concentrations below the critical yield as was the methionine.

Nevertheless, since the methionine content of protein was about equal to the cyst(e)ine content in Experiment 21, the sulphur impurity in this experiment was not large enough to supply all the cysteine required for protein and so cysteine was synthesized from the added methionine.

FIGURE 4-14



THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH HOMOCYSTINE. MEDIUM CONTAINED ION EXCHANGED SUCROSE EXCEPT FOR HISTOGRAMS 'c', THE MEDIUM OF WHICH CONTAINED UNTREATED SUCROSE. HISTOGRAMS: MEDIUM WITH SULPHATE, a - 8 μg. S / 25 ml., b - 16 μg. S / 25 ml., c - 117 μg. S / 25 ml..

Experiment 14

The growth of tomato roots with homocystine

Since homocysteine was considered by Wilson (1962) to be an intermediate in the conversion of cysteine to methionine in microorganisms and since Turner and Shapiro (1961) demonstrated synthesis of methionine from homocysteine in extracts of seeds, homocystine was tried as a source of sulphur to tomato roots. Homocystine rather than homocysteine was supplied as the latter would have been oxidized to the former in the medium.

Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either homocystine or sodium sulphate. A treatment containing untreated sucrose and sodium sulphate was also prepared. The pH of the media was 4.9.

'High magnesium' medium (VIII)

- containing ion exchanged sucrose with
  either 1, 2, 4, 8, 16, 32 µg. homocystine-S/25 ml.
  or 0, 8, 16 µg. sulphate-S/25 ml.

- containing untreated sucrose
  with 117 µg. sulphate-S/25 ml.

Results and Discussion

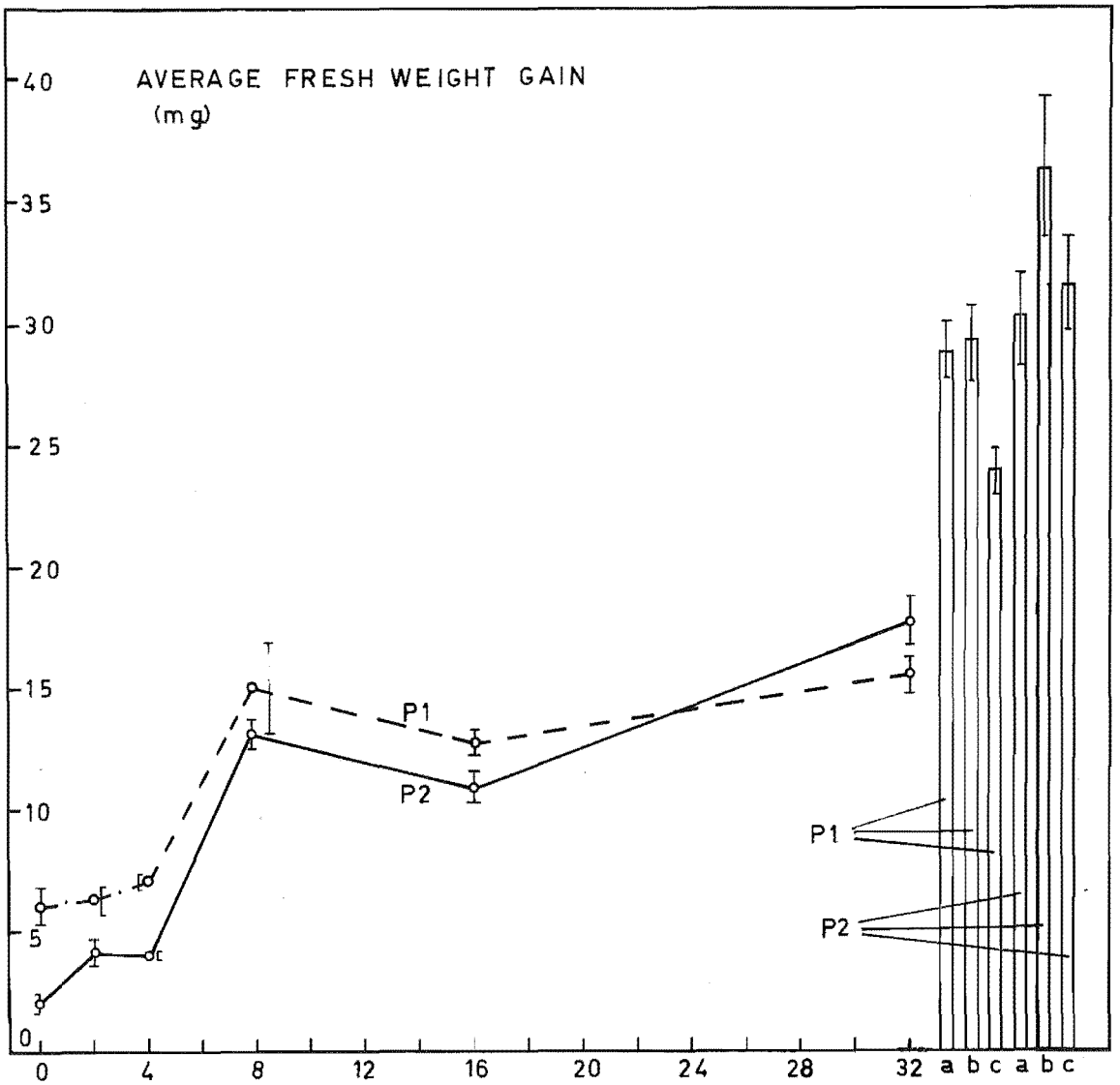
The fresh weight gains shown in Figure 4-14 (Appendix Table 5-13) increased with the addition of homocystine to a maximum of about 45 per cent of the sulphate-sufficient treatments for the first passage and 25 per cent for the second passage, growth in the second passage being less than that in the first. This together with the slight depression of growth at 32 µg.S/25 ml. suggested that homocystine was somewhat toxic under these conditions.

Homocystine is most likely of comparable stability to cystine in solution, since it differs by one in the chain length and its solubility in water is similar. The hydrolytic cleavage of disulphides was considered by Rosenthal (1955) to be related to the basicity of the

molecule. Cystine is abnormally acidic for a disulphide so tending to be cleaved at lower pH values than the others. The acidic nature was also considered by Rosenthal to be responsible for the small solubility, a property shared by homocystine.

Homocystine was therefore assimilated by the roots, although not very rapidly. The amount of growth was only just sufficient in the second passage to conclude that both cysteine and methionine were formed from it, a conclusion which also follows from the utilization of both methionine and cystine and the synthesis of each from the other. This suggests that homocysteine is an intermediate in the interconversion of cysteine and methionine by tomato roots as it is in fungi.

FIGURE 4-15 i



μg. CYSTINE — S/25 ml. MEDIUM.

THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER CYSTINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR (c). HISTOGRAMS: (a) 8; (b) 16; (c) 117 μg. SULPHATE — S/25ml.

Experiment 15

The growth of tomato roots with cystine and with possible cysteine-forming compounds: S-methyl-L-cysteine and glutathione, as well as with cystamine - a compound possibly derived from cysteine

(a) Growth with cystine

Following the addition of cysteine to the medium in Experiment 10, the roots grew optimally at 32 µg.S/25 ml. so the same concentrations of cystine-S were added in this experiment, since the affect of removing the sulphate impurity present in Experiment 10 was unknown. It can be assumed that the added cysteine in Experiment 10 was oxidized to cystine by air before the roots were inoculated into the medium.

Experimental details

To sulphate-omitted 'high magnesium' medium (VIII) containing ion exchanged sucrose was added cystine or sodium sulphate as stated below. A treatment containing untreated sucrose and sodium sulphate was included. The pH of the media was 4.9.

'High magnesium' medium (VII)

- containing ion exchanged sucrose with
either 1, 2, 4, 8, 16, 32 µg. cystine-S/25 ml.
or 0, 8, 16 µg. sulphate-S/25 ml.
- containing untreated sucrose
plus 117 µg. sulphate-S/25 ml.

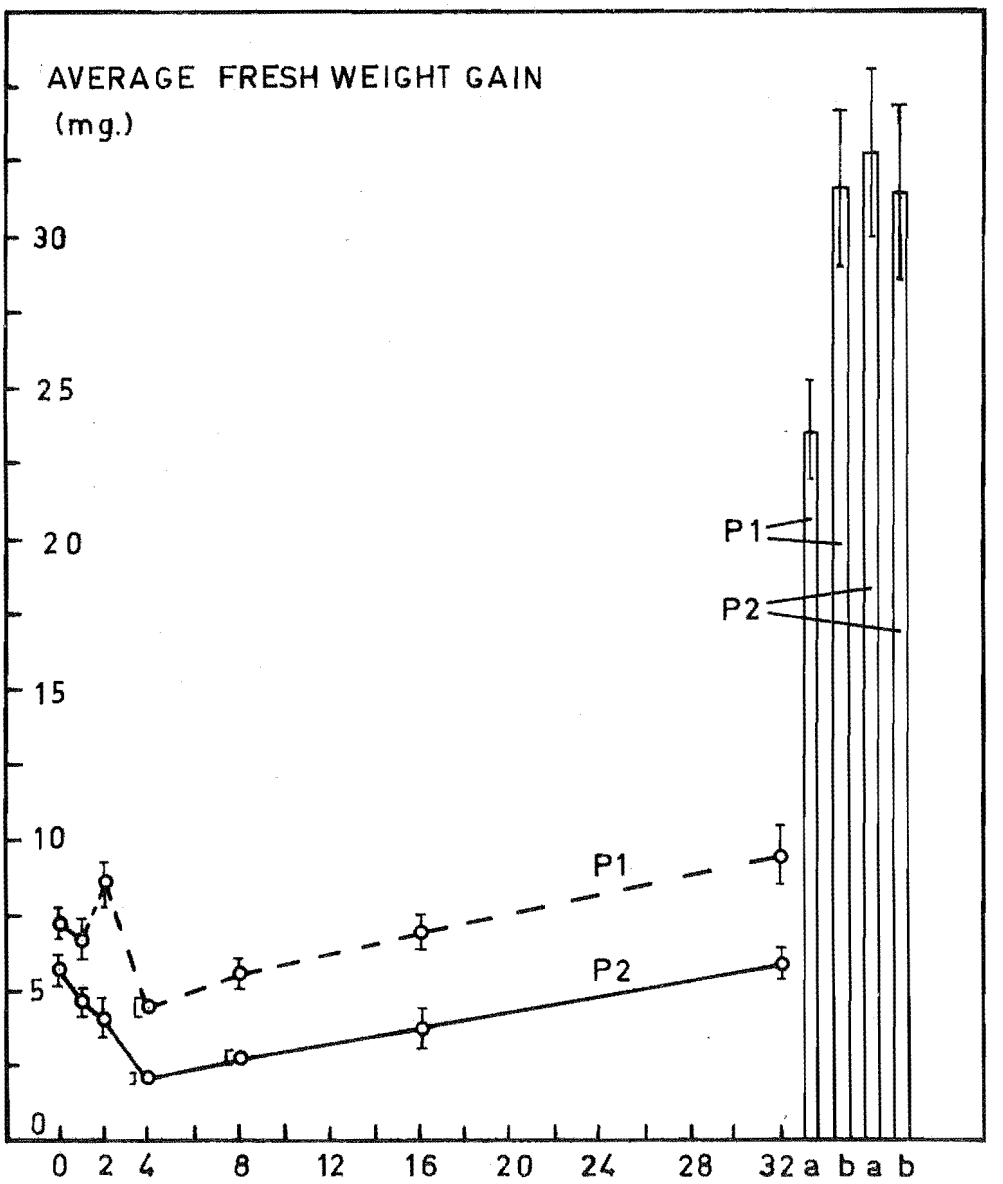
Results and Discussion

The fresh weight gains of tomato roots shown in Figure 4-15i (Appendix Table 5-13) were increased by the addition of cystine only up to nearly one-half of those in sulphur sufficient medium. This poor response compares unfavourably with that to methionine or sulphate so the experiment was repeated with a sample of cystine obtained from Sigma Chemical Co. in place of that used in this part of the experiment which had been obtained from L. Light and Co.. The Sigma sample of cystine was claimed to be of high purity which would help to preclude either the poor growth response being brought about by a toxic impurity or

some impurity being responsible for the growth.

The concentrations used later were increased above those of this first part to establish whether the roots would grow as well with cystine as they did with sulphate, since, if they did and a plateau was reached, the poor response to cystine was more likely to be a result of slow assimilation rather than an inhibition of growth by cystine.

FIGURE 4-15 ii



THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER CYSTEAMINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR TREATMENT (b). HISTOGRAMS: (a) 16μg. (b) 117μg SULPHATE — S/25ml.

Experiment 15

(b) Growth with cystamine

Cystamine ($HCH(NH_2)CH_2$-S-S-$CH_2CH_2(NH_2)$) is similar to cystine in being a disulphide and differs only in lacking the two carboxyl groups. As it might be formed from cystine in animals or from cysteine through coenzyme A (Young and Maw, 1958; Kun, 1961), and it may also be oxidized to sulphate in animals it was tried as a sulphur source in view of the poor assimilation of cystine and homocystine. Penicillium notatum was reported by Hockenhull (1948) to grow in medium to which cysteamine had been added. In this experiment cysteamine (the thiol compound) was added as it would have been oxidized to cystamine in the four days before the roots were inoculated into the medium.

Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either cysteamine or sodium sulphate. A treatment containing untreated sucrose with sodium sulphate was included. The pH of the media was 4.9.

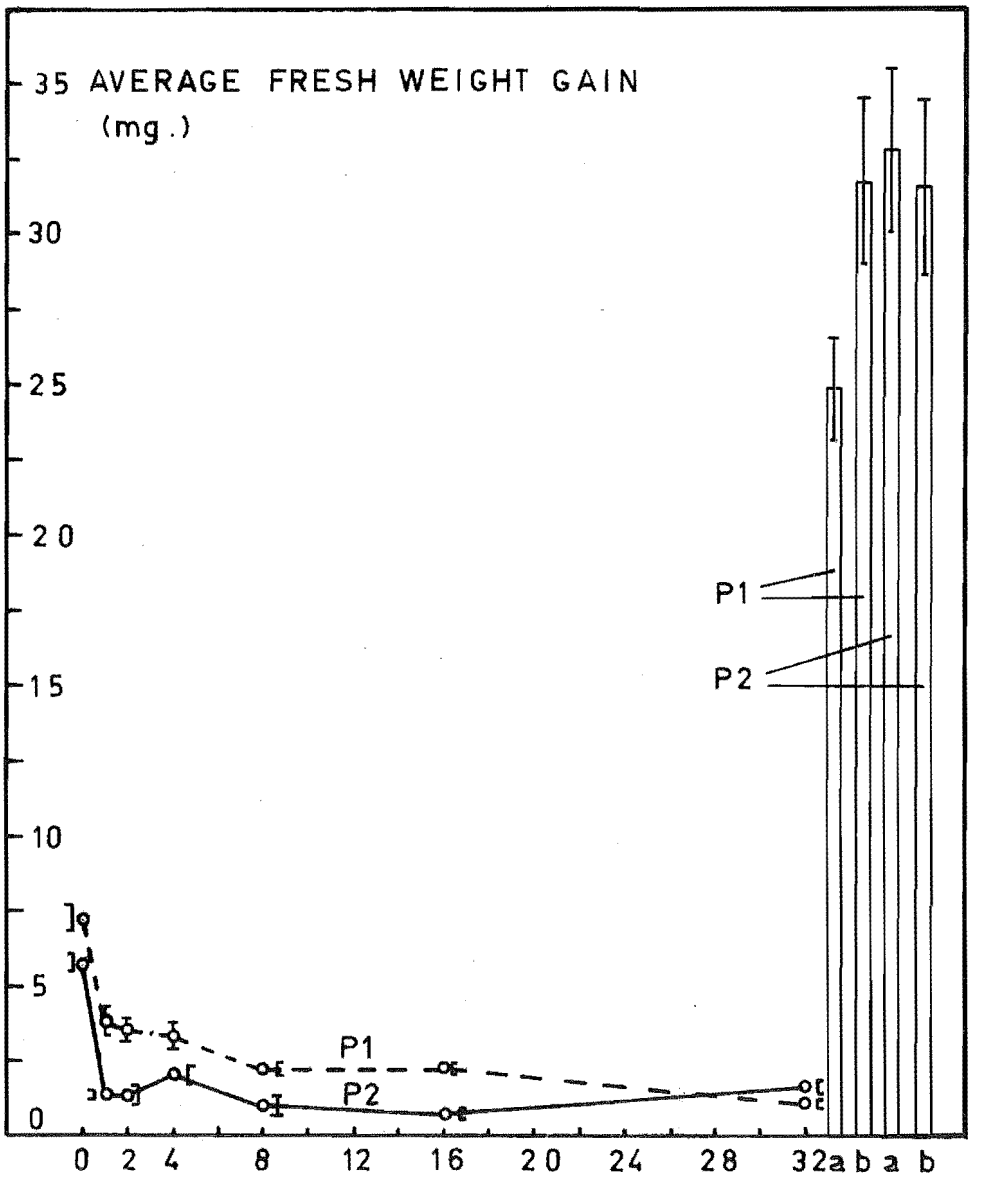
'High magnesium' medium (VIII)

- containing ion exchanged sucrose with
either 1, 2, 4, 8, 16, 32 µg. cysteamine-S/25 ml.
or 0, 16 µg. sulphate-S/25 ml.
- containing untreated sucrose
plus 117 µg. sulphate-S/25 ml.

Results and Discussion

The fresh weight gains of the tomato roots shown in Figure 4-15ii. (Appendix Table 5-16) mostly declined until 4 µg.S/25 ml. had been added and then slightly increased up to a level at 32 µg.S/25 ml. comparable with that of the minus sulphate treatment. In the second passage the increase of fresh weight was less than in the first. It was concluded from these results that cystamine was not assimilated by tomato roots.

FIGURE 4-15 iii



μg. S-METHYLCYSTEINE — S/25ml. MEDIUM
THE GROWTH OF TOMATO ROOTS IN HIGH MAGNESIUM MEDIUM WITH EITHER S-METHYLCYSTEINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR (b). HISTOGRAMS: (a) 16; (b) 117μg. SULPHATE-S/25ml.

Experiment 15

(c) Growth with S-methylcysteine

Since methionine which is the S-methyl derivative of homocysteine was assimilated in Experiment 13 and since S-methylcysteine - the comparable derivative of cysteine - was concluded by Thompson and Gering (1966) to be formed from cysteine in radish, its assimilation by tomato roots was investigated in case it gave rise to cysteine by demethylation. If it formed cysteine and was assimilated it would be interesting to compare the efficiency of its utilization with that of cystine to help find out why cystine was so slowly assimilated.

Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either S-methylcysteine or sodium sulphate. A treatment containing untreated sucrose with sodium sulphate was included. The pH of the media was 4.9.

'High magnesium' medium

- containing ion exchanged sucrose with
either 1,2,4,8,16,32 µg. S-methylcysteine-S/25 ml.
or 0, 16 µg. sulphate-S/25 ml.
- containing untreated sucrose
plus 117 µg. sulphate-S/25 ml.

Results and Discussion

The fresh weight gain of tomato roots in sulphate-omitted medium, shown in Figure 4-15iii (Appendix Table 5-16) was inhibited by the addition of S-methylcysteine and the main axis ceased growing before the end of the first passage. In the second passage many short thin laterals were produced at concentrations greater than 2 µg.S/25 ml. while at 32 µg.S/25 ml. the tips had turned brown by the end of the passage. From these results it was concluded that S-methylcysteine was unavailable for growth of tomato roots and thus did not give rise to cysteine.

Experiment 15

(d) Growth with glutathione

Although tomato roots assimilated cystine in Part (a) of this experiment, the growth response to this compound was less than that to either sulphate or methionine at equivalent S concentration. Maw concluded in his 1965 review that Saccharomyces yeasts utilized glutathione, as well as they did methionine and more readily than they did cyst(e)ine. Fejer (1957) found that the xylem sap of corn plants contained glutathione in quantities similar to methionine and which reached a maximum of 8 per cent of the total sulphur during the growing season. Present results with tomato roots labelled with radiosulphate indicated that glutathione was present in quite large amounts so it seemed possible that glutathione might be assimilated more readily than cystine. If it were utilized, it would be expected, from the results of Binkley (1961), to be hydrolyzed into its constituent amino acids, so that it would be the equivalent of cysteine in relation to the pathway of sulphur assimilation.

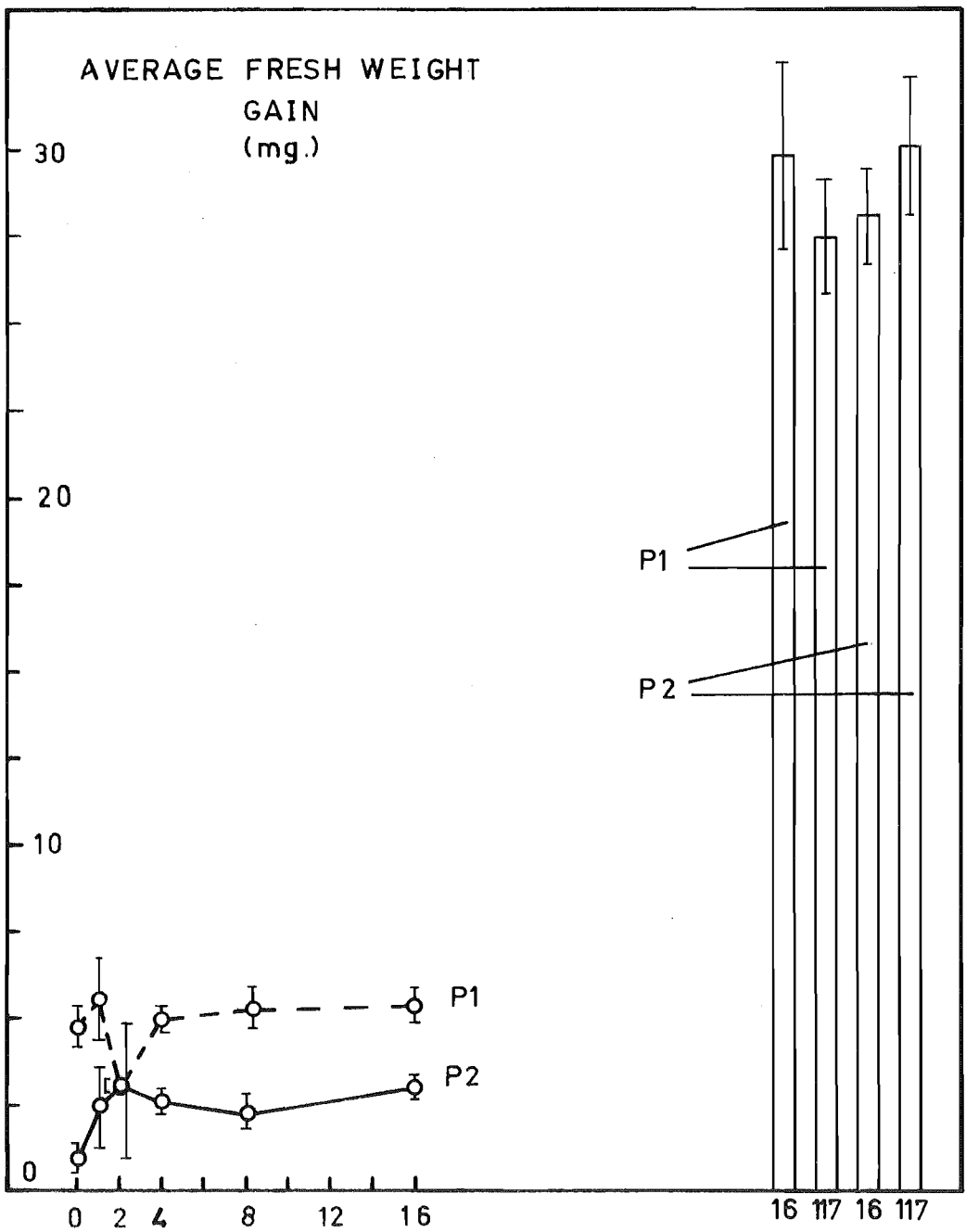
Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either reduced glutathione, which would rapidly oxidize to the disulphide, or sodium sulphate. A treatment containing untreated sucrose with sodium sulphate was included. The pH of the media was 4.9.

'High magnesium' medium (VIII)

- containing ion exchanged sucrose with
  either 1, 2, 4, 8, 16 µg. glutathione-S/25 ml.
  or 0, 16 µg. sulphate-S/25 ml.
- containing untreated sucrose
  with 117 µg. sulphate-S/25 ml.

FIGURE 4–15 iv

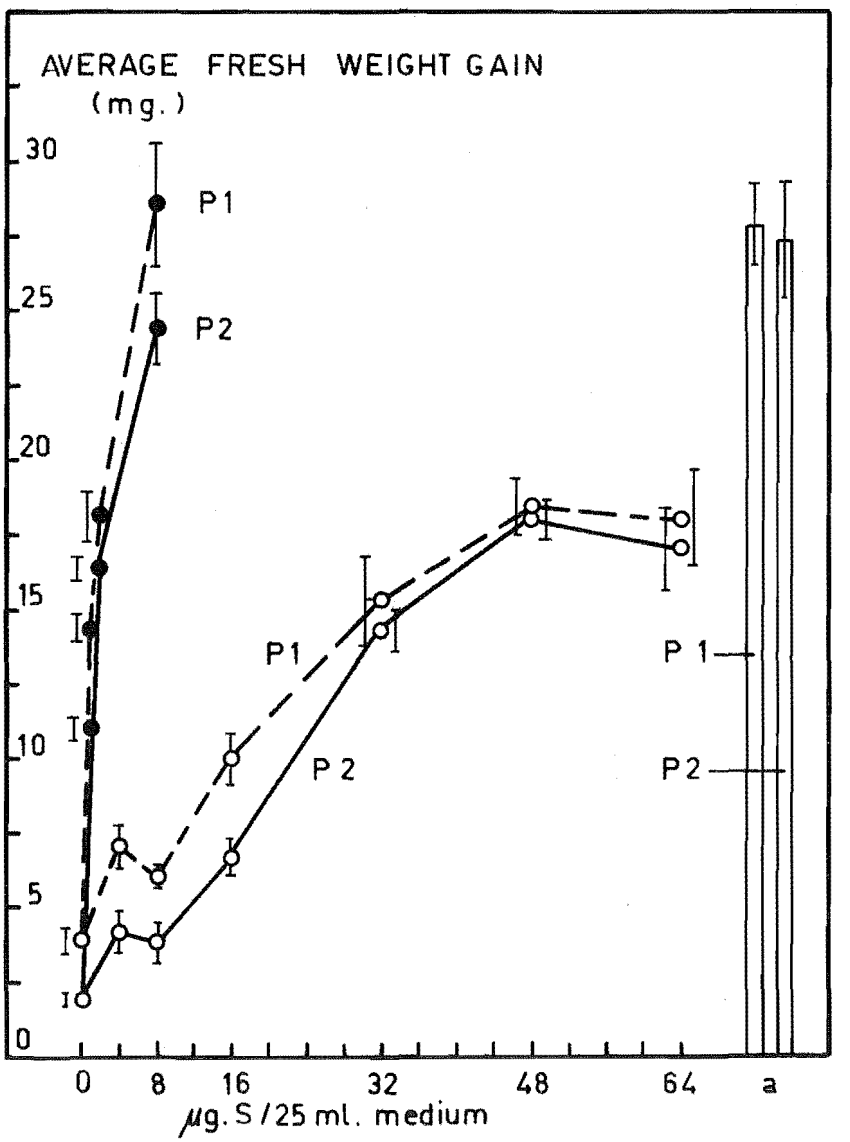


THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER SULPHATE OR GLUTATHIONE. SUCROSE WAS ION EXCHANGED EXCEPT FOR THE 117µg. SULPHATE–S TREATMENT.

Results and Discussion

The fresh weight gains shown in Figure 4-15iv (Appendix Table 5-17) were unaffected in the first passage by the addition of glutathione. In the second passage all the treatments containing glutathione had greater fresh weight gains than the minus-sulphate treatment, but this apparent response of the roots to glutathione did not increase with the increase of glutathione concentration. The fresh weight gain of the minus-sulphate control was lower than usual as it was 0.94 mg. compared with a usual range of 2 to 3 mg. Since the treatments containing glutathione lay within this range it was concluded that the tomato roots did not assimilate glutathione as a source of sulphur.

FIGURE 4-15 v



THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER CYSTINE OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR TREATMENT 'a'. HISTOGRAMS: a- 117µg. S / 25 ml.. CURVES: o—o, o- -o CYSTINE; ●—● ●- -● SULPHATE.

Experiment 15

(e) Growth with cystine

Since the efficiency of utilization of cystine was low in Part (a) and the presence of sulphate or a toxic impurity could have explained the results, a purer sample of cystine, obtained from 'Sigma', was used for this experiment. The maximum growth with cystine in Part (a) of this experiment was about 40 to 50 % of that with sulphate and to establish whether a suboptimal plateau or a peak would be reached if maximum growth with cystine did not equal that with sulphate, the higher concentration of cystine was increased from 32 to 64 µg.S/25 ml.

Experimental details

To 'High magnesium' medium (VIII) containing ion exchanged sucrose was added either cystine or sodium sulphate. A treatment of untreated sucrose with sodium sulphate was included. The pH of the media was 5.0.

'High magnesium' medium (VIII)

- containing ion exchanged sucrose with
  either 4, 8, 16, 32, 48, 64 µg. cystine-S/25 ml.
  or 0, 1, 2, 8 µg. sulphate-S/25 ml.

- containing untreated sucrose
  plus 117 µg. sulphate-S/25 ml.

Results and Discussion

The fresh weight gain shown in Figure 4-15v (Appendix Table 5-15) was increased up to 65 per cent of the maximum in sulphate by the addition of between 48 and 64 µg.S/25 ml. The growth response in the 8 µg.S/25 ml. treatment was lower than that in Part (a) but the curves were consistent in that the response is poor compared with that to sulphate; for instance cystine at 48 µg.S/25 ml. in Figure 4-15v gave equivalent growth to sulphate at 2 µg.S/25 ml. in Figure 4-11. The difference of growth response to cystine at 8 µg. S/25 ml. in the two parts of this experiment was not important since the curve in this part is more or less straight while the curve in Part (a) would also have been straight but for the bump in the curve at the 8 µg.S/25 ml.

The response curve in this part of the experiment reached a peak or a plateau - the two cannot be distinguished from these results - at about 70 per cent of the optimum fresh weight gain with sulphate. However an inhibition of growth by cystine did not explain the low efficiency of its assimilation since growth was quite good at 64 μg.S/25 ml. which was 16 times that required, except in the unlikely event that cystine inhibited its own uptake. Regardless of the low efficiency, the addition of cystine stimulated growth sufficiently, compared with the sulphate-omitted treatment, to conclude that it was assimilated for growth.

The termination of the curve, provided that it was not brought about by inhibition, is most likely explained by either cystine supplying only part of the sulphur needs or by its rate of assimilation limiting growth. Since the sulphur impurity present in the medium was sufficient for 2 to 3 mg. fresh weight gain and from Experiment 21 cysteine constituted about half of the protein sulphur then protein methionine must have been formed from cystine for the roots to have increased 17 mg. in fresh weight.

Discussion of the growth of tomato roots with sulphur compounds

From the growth of excised tomato roots in sulphur deficient media, with cystine or methionine as sulphur sources, it was concluded that both of these amino acids can be converted into the other and used for protein synthesis. Since homocysteine is an intermediate in the conversion of one to the other in bacteria and fungi, the slight growth response of roots to this compound was consistent with its being such an intermediate in higher plants. The failure of the roots to grow with cysteic acid, taurine, cystamine, S-methylcysteine and glutathione does not indicate whether the roots metabolize these compounds since they might have failed to take them up.

The available evidence for higher plants, which is discussed in the Introduction, suggests that the following pathway operates to assimilate sulphate into methionine:

sulphate	→	cysteine	→	cystathionine	→	homocysteine	→	methionine
SO_4"		3C-SH		3C-S-4C		HS-4C		CH_3-S-4C

The assimilation of cystine was consistent with such a pathway because after reduction to cysteine it would be used in protein synthesis and after conversion to methionine as above, it would also supply methionine for protein synthesis.

The growth of the roots with methionine was likewise consistent with the fungal and mammalian pathway:

methionine	→	homocysteine	→	cystathionine	→	cysteine	
4C-S-CH_3		4C-SH		4C-S-3C		HS-3C	

(α-ketobutyrate)

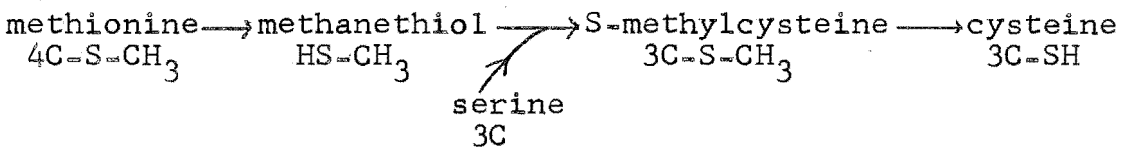
However Giovanelli and Mudd (1966a) suggested that since crude extracts of spinach catalyzed the degradation of cystathionine to pyruvate (and therefore to homocysteine) much faster than they did to α-ketobutyrate (and therefore to cysteine) the mammalian and fungal transsulphuration from homocysteine to cysteine may not be present in higher plants.

As an alternative, tomato roots might degrade homocysteine or homocystine to inorganic sulphur and then assimilate the latter into cysteine.

$$\text{methionine} \xrightarrow{(a)} \text{homocysteine} \rightarrow \text{homocystine} \xrightarrow{(b)} S^{o} \rightarrow S'' \rightarrow SO_3'' \rightarrow SO_4''$$

In support of (a) Doney and Thompson (1966) found that in turnip leaves (^{35}S) methionine was converted into homocysteine. For (b) an enzyme from cabbage leaves degrading cystine to elemental sulphur (Tishel and Mazelis, 1966) also degraded homocystine. The elemental sulphur might then react non enzymatically with thiols to form sulphide as in animal tissues (Sluiter, 1930) which would be oxidized to sulphate as found by Wetter (1964). The sulphate would be reduced and incorporated into cysteine. It is possible that cysteine could by synthesized directly from sulphide which was formed from sulphur but the failure of Wetter (1964) to achieve any significant labelling of the isothiocyanate group of sinigrin and hence methionine is inconsistent with this.

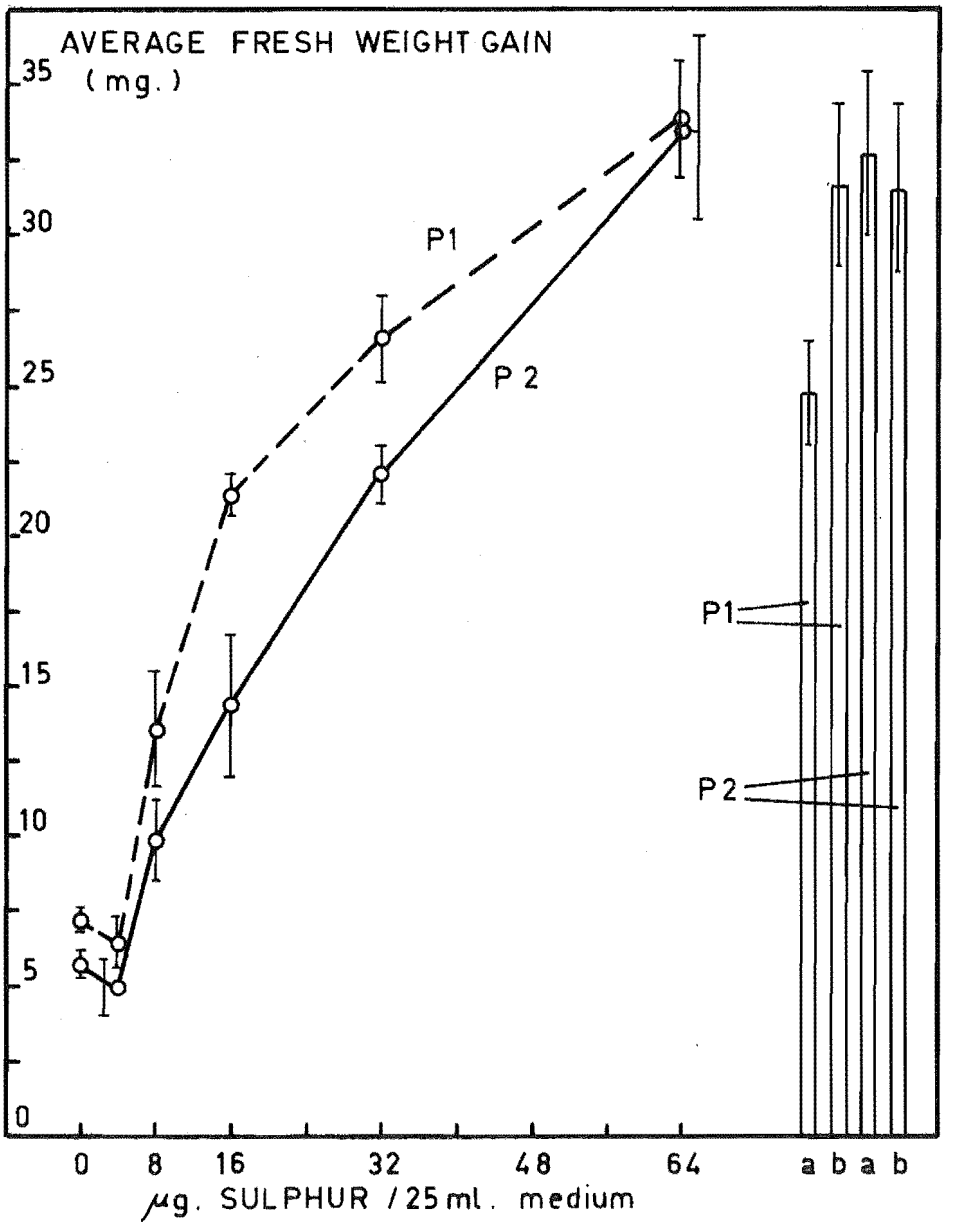
Methionine could also be converted into S-methylcysteine as suggested for garlic by Sugii et al. (1963) and the latter demethylated to form cysteine.



However Thompson and Gering (1966) found that this pathway did not operate in radish leaves which contained S-methylcysteine.

To establish which of these pathways operate in tomato roots, (^{35}S) methionine was fed to roots and the extracts of these examined for labelled homocysteine, cystathionine or S-methylcysteine. The radioactivity present in sulphate was also to be measured so that the likelihood of methionine degradation to sulphate could be determined.

FIGURE 4—16.



THE GROWTH OF TOMATO ROOTS IN 'HIGH MAGNESIUM' MEDIUM WITH EITHER SULPHUR OR SULPHATE. SUCROSE WAS ION EXCHANGED EXCEPT FOR TREATMENT 'b'. HISTOGRAMS: a:- 16, b:- 117 µg. SULPHATE - S / 25ml.

Experiment 16

(a) The growth of tomato roots with elemental sulphur

Radioactive elemental sulphur was claimed by Turrel and Weber (1955) to label the proteins of lemon leaves on to which it had been dusted, so it was tried as a source of sulphur in Experiment 10, but the results were equivocal, for if the elemental sulphur was assimilated then the response of the roots was small. In this experiment the concentration of sulphur was doubled in case assimilation, if there was any, was again poor.

Experimental details

To 'high magnesium' medium (VIII) containing ion exchanged sucrose was added either elemental sulphur in colloidal solution, prepared as in Methods and Materials (3-6) or sodium sulphate. A treatment containing untreated sucrose with sodium sulphate was also included. The pH of the media was 4.9.

'High magnesium' medium

- containing ion exchanged sucrose with
either 4, 8, 16, 32, 64 µg. elemental S/25 ml.
or 0, 16 µg. sulphate-S/25 ml.

- containing untreated sucrose
with 117 µg. sulphate-S/25 ml.

Results and Discussion

The fresh weight gains shown in Figure 4-16 (Appendix Table 5-16) were markedly increased by the addition of elemental sulphur to the medium. Growth equivalent to the optimum in sulphate was given by 32-64 µg.S/25 ml., so it was about one-eighth as efficient as sulphate in stimulating growth.

It cannot be concluded from this that elemental sulphur was assimilated since oxidation of the sulphur could have occurred either in the medium or while it was stored in acetone at 5°C. That the latter was possible was shown by the presence of sulphur anions in a sample of radioactive elemental sulphur in chloroform which was examined in Experiment 22 (a). Oxidation of the sulphur during the growth

period seemed unlikely as growth in the second passage, which was prepared at the same time as the first and stored at room temperature, was less than in the first except at the highest concentration of 64 µg.S/25 ml. If oxidation of the sulphur in the medium was continuous, the growth at the lower sulphur concentrations would have been greater in the second passage as these contained sulphur in excess of that needed for optimum growth. It was however possible that only a portion of the colloid was oxidized because of its particle size being smaller than the bulk of the preparation.

It was most likely that the elemental sulphur would have been oxidized during storage or during the preparation of the sterile colloidal solution since in the latter the sulphur was sterilized by autoclaving, which Mellor (Vol. X, p. 92, 1930) reported to increase the hydrolysis of sulphur. The medium was therefore examined in the next part of this experiment for sulphur anions which could supply the sulphur requirements of roots and which were formed during the preparation of the medium.

Experiment 16

(b) The growth of tomato roots in medium containing the chloroform insoluble portion of the autoclaved sulphur sol.

In the first part of this experiment the addition of an autoclaved sol of elemental sulphur to the medium increased the fresh weight gain of tomato roots. The roots could have grown with either the sulphur or with anions formed by oxidation of the sulphur either in the acetone solution or during the preparation and autoclaving of the sol.

To decide whether the elemental sulphur was oxidized during preparation of the medium, the sol of elemental sulphur, after autoclaving in water, was extracted with chloroform and the residual solution tested as a source of sulphur. It was shown in Experiment 22 (a) that sulphur oxidation products are not removed by this procedure, so any sulphate produced from the oxidation of sulphur will be added to the medium. For comparison with this treatment, a treatment containing elemental sulphur at 64 µg.S/25 ml. was included. This concentration was also used for the sol which was extracted with chloroform since this gave optimum growth in first part of this experiment and the greater the quantity of sulphur the greater should have been the quantity of an oxidized impurity.

Experimental details

A colloidal solution of elemental sulphur was autoclaved in bulk (105 ml. of water containing 64 µg.S/5 ml. for 21 flasks of medium) at the concentration used in the 64 µg.S/25 ml. treatment, and then extracted five times with 20 ml. portions of chloroform and purified in a 250 ml. separating funnel as stated below. The solution was boiled to expel any remaining chloroform and added to the bulk of the medium which was then autoclaved.

A quantity of water (105 ml.) was autoclaved at the same time as the elemental sulphur sol and extracted with chloroform in the same way. The aqueous layer was then added to the bulk of the medium containing 16 µg. sulphate-

S/25 ml. to test for any inhibition of growth by the chloroform treatment.

The other treatments were prepared as usual to give the concentrations shown below. The pH of the media was 4.9.

Purification of the chloroform

The first time this experiment was carried out, growth in the chloroform treated water and sulphate treatment was little more than that in the medium with extracted colloidal sulphur (Appendix Table 5-17) so the chloroform was purified as follows.

Although some chloroform had remained on the water when it was added to the bulk of the medium in the abortive experiment, this should have disappeared on autoclaving. The water extracts were boiled this time before adding the medium in case the chloroform had not evaporated. 'Pronalys' chloroform (300 ml.) was distilled at 64-65°C. The first 50 ml. of distillate was discarded and about 100 ml. of chloroform was left in the boiling vessel. Then 200 ml. of the distilled chloroform was washed with 5 l. of double distilled water in a long glass tube by allowing the water to enter at the bottom and siphon off at the top. This washed chloroform was used to extract the elemental sulphur.

'High magnesium' medium (VIII)

with ion exchanged sucrose

	Treatments					
	water or sol extracted with chloroform			media prepared as usual		
µg. elemental-S/25 ml. and	64	0		64	0	0
µg. sulphate-S/25ml.	0	16		0	16	0

with untreated sucrose

plus 117 µg. sulphate-S/25 ml.

Table 4-16

'High magnesium' medium (VIII)

containing ion exchanged sucrose

	µg.S/ 25 ml.	Average Fresh Weight gain (mg.) First Passage	No.	Second Passage	No.
- plus sulphate	0	6.4±1.0	10	3.7±0.9	9
	16	25.6±0.9	9	29.0±2.5	9
and 100 ml. water extracted with chloroform	16	26.5±1.0	10	30.5±1.8	9
- plus elemental sulphur	64	25.2±1.2	10	20.8±1.0	8
- plus elemental sulphur sol extracted with chloroform	0	7.1±0.5	10	4.7±0.4	10
containing untreated sucrose					
- plus sulphate	117	26.3±0.8	10	28.7±1.9	10

Results and Discussion

The fresh weight gains in Table 4-16 (Appendix Table 5-18) show that extraction of the water with chloroform does not inhibit growth and that growth in medium containing water soluble constituents of elemental sulphur sol was little more than that in the minus sulphate control. Therefore the utilization of elemental sulphur does not depend upon oxidation to sulphate in the sterilization of the sol, but the possibility still remains that it is oxidized in the medium.

Even if elemental sulphur was assimilated as such, it was not known whether the sulphur would have been oxidized to sulphate before assimilation into proteins. If no sulphate was formed during uptake of radioactive elemental sulphur then it would have been established that it was assimilated of itself, so a tracer experiment was carried out first before the possibility of sulphur oxidation in the medium was again investigated.

Experiment 17

The establishment of conditions for the labelling of sulphur compounds with (^{35}S) sulphate

When about 0.8 g. fresh weight of Petkus rye seedlings was supplied for one, two, four, and eight hours, with 10 μC. of carrier free sulphate plus 10 μg. sulphate-S, paper chromatography of the root extracts revealed that three or four spots were labelled after two hours and that their intensity increased after four and eight hours without any further spots appearing. It seemed from this that the sensitivity was so low that other compounds might be present which did not make a visible image on the film.

In this experiment rye seedlings were exposed for three hours to 50 μC. of carrier free sulphate, so that a greater amount of labelling of sulphur compounds would take place. Although carrier free radiosulphate is adsorbed to glass, the presence of an excess of phosphate, which is strongly adsorbed also counteracted this tendency.

Experimental details

Seeds of rye were aerated and soaked for four hours in a sintered glass funnel to promote even germination. The soaked seeds were placed groove side down on Nylon netting suspended 6 mm. above distilled water and germinated for four days in front of a window at room temperature. About seven of these seedlings were placed inside a polystyrene ring floating on modified Bonner's medium ('rye clone' medium minus iron, sugar, vitamins and micronutrients), and left at room temperature for one day. To increase the rate of metabolism, the seedlings were placed in an incubator at 25°C. for 12 hr. prior to the experiment in fresh modified Bonner's medium.

They were then supplied 50μC. carrier free (^{35}S) sulphate for three hours in 20 ml. of modified Bonner's medium contained in a 50 ml. beaker at 25°C.

The seedlings were taken out and the roots cut off. The roots were blotted, weighed and pushed into a 50 ml. beaker containing 10 ml. 80% ethanol as well as dry ice.

FIGURE 4-17

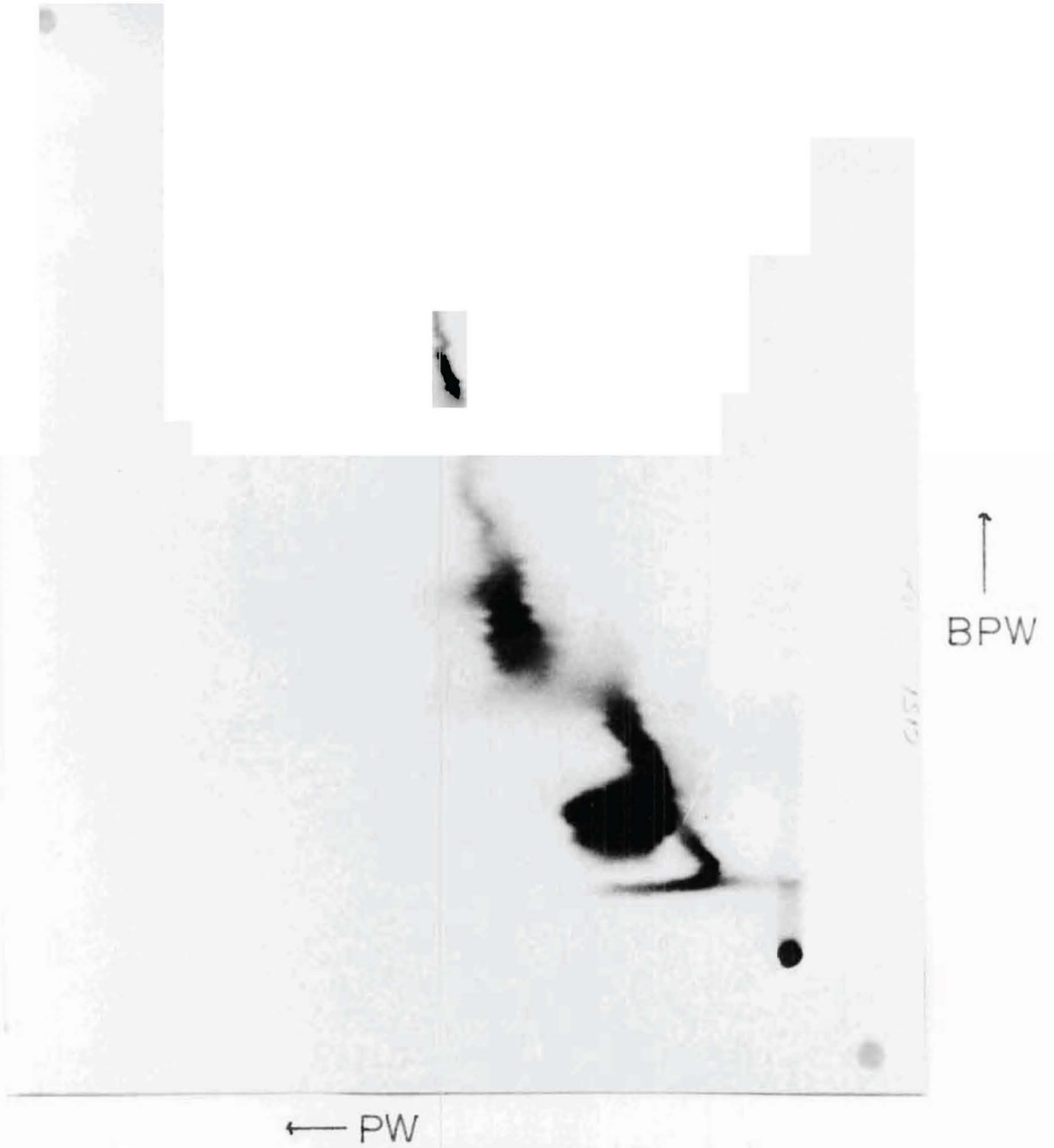


FIGURE 4-17. AUTORADIOGRAM OF RYE ROOT EXTRACT AFTER CHROMATOGRAPHY WITH BPW FOLLOWED WITH PW. ROOTS WERE INCUBATED WITH (^{35}S) SULPHATE FOR 3 HR..

The temperature of the extract and roots was allowed to rise to -15°C. at which they were stored.

The roots were homogenized and extracted as described in Methods and Materials (3-10) except that 80% ethanol was used to homogenize the roots and the final double distilled water rinse was omitted. After evaporation of the extract it was taken up in 0.5 ml. of 10% isopropanol and stored at less than -15°C.

Results and Discussion

Figure 4-17 shows that many compounds became visible in the autoradiogram of a two dimensional chromatogram. It was concluded from this that the use of carrier free (^{35}S) sulphate was desirable to increase the efficiency of detection of labelled compounds. So many compounds appeared that it was impossible to conclude which were the earlier formed ones and a shorter time of exposure was indicated.

Many of the compounds labelled have streaked badly during chromatography, and this made identification difficult and comparison between chromatograms almost impossible so that it was necessary to improve the separation of these sulphur compounds. This is more fully discussed at the end of Experiment 18.

Experiment 18

The assimilation of (^{35}S) sulphate by tomato roots and the stabilization of thiol compounds

In Experiment 17, an extract of rye seedling roots contained many labelled compounds after they had assimilated carrier free radiosulphate for three hours. If the time of exposure to the radioisotope was reduced, those spots which were more directly related to sulphate would retain a larger amount of radioactivity so a shorter exposure of one hour was used. At the same time non-sterile rye seedlings were replaced with excised roots of tomato, which were then being cultured for nutritional experiments, so that labelling of compounds could be unequivocally ascribed to the metabolism of the roots.

When tomato roots were killed in 80% ethanol-dry ice mixture, the extract and residue turned dark brown and contained substances, possibly polyphenols from the colour, which seemed to cause streaking of the extract in chromatography. As freezing does not always inactivate enzymes tomato roots were killed with the MCF extractant of Bieleski and Young (1963) and this procedure was found to markedly reduce the forming of a brown colour. Nearly all enzymes are inactivated by heat but it was considered that boiling the roots with ethanol would degrade or oxidize some of the sulphur compounds.

After thiols are extracted from tissue, they become oxidized by the air to disulphides and sulphonic acids. The disulphides formed are frequently mixed (Eldjarn and Pihl, 1956), (for example the combination of glutathione and cysteine is a mixed disulphide), and these increase the number of radioactive spots without any increase in radioactive compounds.

The mixed disulphides can be later split into their respective parts by oxidation with hydrogen peroxide, to the sulphonic acids. However oxidation with 30% hydrogen peroxide of a known mixture of sulphur amino acids produced a positively charged compound at pH 5.3 which was not

expected from those compounds present. Since oxidation of extracts might also produce artifacts the protection of thiol groups from oxidation was tried, so preventing them from forming mixed disulphides.

Oxidation of thiol or sulphydryl groups can be prevented by coupling them with either N-ethylmaleimide (NEM) or iodoacetamide. The adducts formed by these reactions are stable and easily chromatographed as they do not streak as disulphides such as cystine tend to (Ellis, 1963; Schiff, 1964). The effectiveness of this procedure depends to a large extent on the position of equilibrium between SH and S-S groups. If the former predominate then reasonable quantities of the adducts are likely to be formed and this was examined in this experiment.

The rate of reaction of these reagents is greater at higher pH values so it appears that the ionized form of the sulphydryl group is the reactive species. The rate for NEM is most rapid in neutral to alkaline conditions but NEM and iodoacetimide are unstable to alkali so the reaction is usually carried out at neutrality.

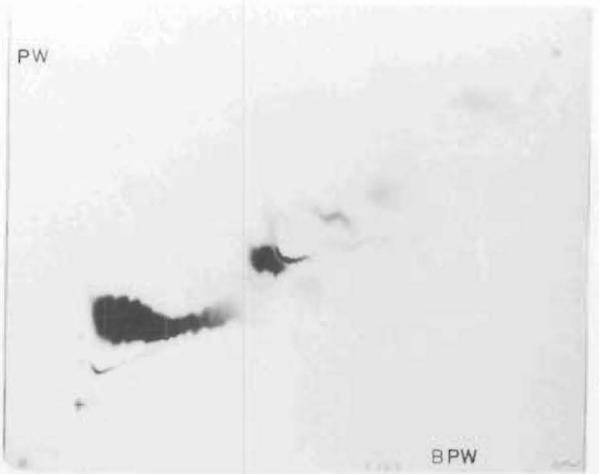
For the control of pH, the MCF was replaced with MCW modified to include the phosphate concentration of the ethanol-phosphate buffer of Ellis (1966) which he used for the extraction and reaction of thiols with NEM. The same buffer (pH 7.4) also seemed suitable for iodoacetamide as Trudinger (1965) reported rapid reaction between it and thiols at this pH.

Experimental details

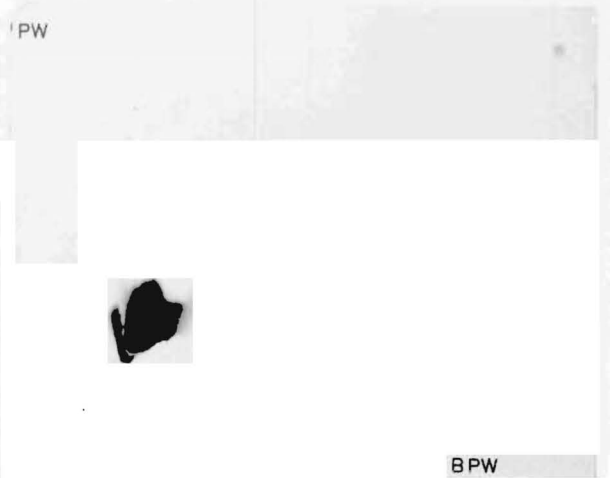
For each treatment, 11 to 12 seven day old tips of tomato were placed in 25 ml. of sterile 'low N' medium (VI).

Fifty μC. of radiosulphate was added and the roots incubated at 26°C. After an hour, they were lifted out of the medium, blotted, weighed and put into MCF or MCP at 5°C. The extractant was left for half an hour at room temperature to allow the NEM and iodoacetamide to react with the thiols and then stored at less than -15°C. until homogenized.

FIGURE 4-18



(i) ROOTS KILLED AND EXTRACTED WITH MCP CONTAINING NEM



(ii) ROOTS KILLED AND EXTRACTED WITH MCP

(iii) ROOTS KILLED AND EXTRACTED WITH MCP CONTAINING IODOACETAMIDE

FIGURE 4-18. AUTORADIOGRAMS OF EXTRACTS FROM TOMATO ROOTS AFTER CHROMATOGRAPHY WITH BPW FOLLOWED BY PW. THE ROOTS WERE INCUBATED WITH (^{35}S)SULPHATE ONE HOUR.

Treatments

tomato roots killed with MCF.

tomato roots killed with MCP containing iodoacetamide.

tomato roots killed with MCP containing NEM.

Results and Discussion

Figure 4-18 shows two dimensional chromatograms of each extract. The spots streaked considerably and this made valueless comparisons between the chromatograms. However a new dense spot appeared in the NEM extract in the region where NEM-cysteine would be expected to run. It seemed from this that sufficient of the sulphydryl compound was present to justify the use of thiol reagents. It was presumed that ACys did not appear because it failed to separate from sulphate, above which it moves in standard mixtures.

Although a satisfactory amount of labelling took place liitle could be learned of the identity of compounds from these chromatograms because of the considerable streaking and distortion of many of the spots. When the extract was spotted on paper it became difficult to dry and the added drop of extract flowed in slowly as though the spot were clogged. From this it was concluded that high molecular weight compounds such as carbohydrates were probably interfering during chromatography.

For the removal of neutral substances from amino acids, ion exchange absorption of amino acids in plant extracts was reported by Plaisted (1958) and Thompson et al. (1959) to allow excellent separation by chromatography of the purified amino acids. Some of the extracts of Experiment 17 and 18 were fractionated by ion exchange as described in Methods and Materials (3-11) and the results are discussed there.

It was found that chromatography was still unsatisfactory for the separation of the compounds present in the neutral amino acid fraction but electrophoresis at pH 5.3 on 11.5 x 11.5 cm. TL plates followed by chromatography in propanol-water separated the compounds into discrete spots. Later the compounds were separated by electrophoresis at pH 2.0 followed by propanol-water chromatography on 20 x 20 cm. TL plates.

Experiment 19

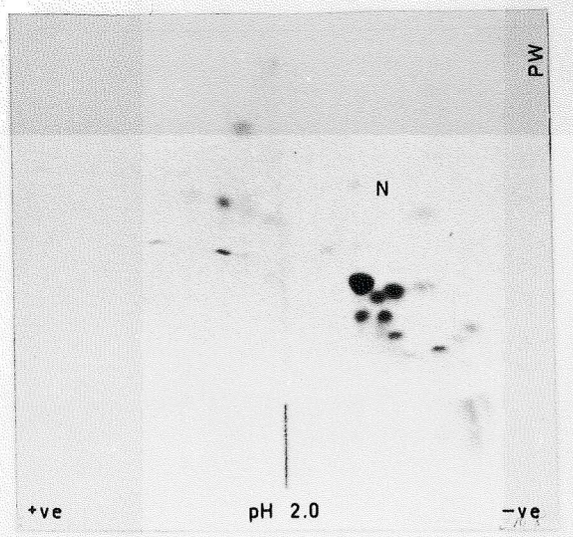
Discussion of reproducibility of the labelling pattern in Experiments 20, 21, and 22

In this series of experiments it was intended to compare the radioactive compounds in extracts from roots which had been treated differently. To be sure that differences were not due to variability between the roots, treatments of 11 or 12 roots were supplied with (^{35}S) sulphate for one hour on three different occassions and the extracts of these were compared.

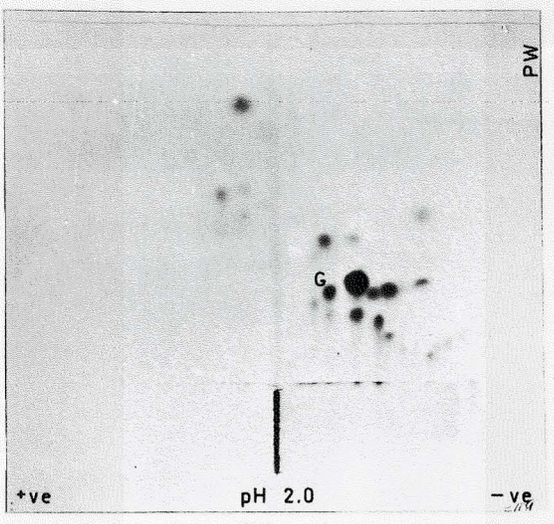
Although the results of this experiment were not complete until after Experiment 22 was finished, they are discussed now because the establishment of differences among the labelling patterns in Experiments 20, 21 and 22 depends upon their not being brought about by chance variations of technique or altered metabolism of the roots.

In the course of these experiments (20, 21 and 22) minor difficulties were overcome by modifications of the technique. Apart from the increase of extractant used in Experiment 22, these modifications were not likely to affect the results of the experiments. The substitution of hydrochloric acid for sodium formate would not have altered the elution of strong anions from the resin, since an excess sufficient to regenerate the column was used, so removing all the remaining anions. The washing of the cation column with MCW in Experiment 22 was also unlikely to alter the compounds labelled since it removed only the lipids which were adsorbed on the resin and some of which appeared in the neutral amino acid fraction (NF). These remained on the start line during electrophoresis. After washing the resin with MCW in Experiment 22 the quantity of these lipid materials in the NF was greatly reduced.

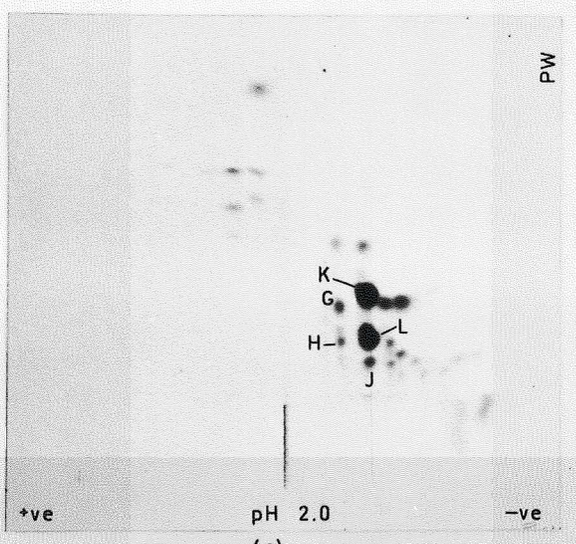
FIGURE 4—19



(a)



(b)



(c)

EXPERIMENT 19. AUTORADIOGRAMS OF NF'S FROM EXTRACTS OF TOMATO ROOTS INCUBATED WITH (^{35}S)SULPHATE FOR ONE HOUR.

Figure 4-19 shows autoradiograms of the neutral amino acids (NF's) of the three [(^{35}S) sulphate 1 hr.] treatments which were also parts of Experiments 20, 21 and 22. The autoradiograms are also shown in those experiments: Figure 4-19 (a) is the same as Figure 4-20iii(a); Figure 4-19 (b) is the same as Figure 4-21ii(a); and Figure 4-19 (c) is the same as Figure 4-22ii(b). Each extract contains some spots not apparent in the others.

Figure 4-19 (a) has the smallest number of positive spots but contains (N) which does not appear in (b) and does in (c). Figure 4-19 (b) has (G) and Figure 4-19 (c) has (G), (H), (J), (N) and also (K) and (L) which overlie spots already present. Figures 4-19 (a) and (c) have more negatively charged spots than (b) although this may have been caused by the smaller amount of radioactivity in (b).

The killing and extracting of the roots and the elution of the NF's for Figure 4-19 (a) was similar to the procedure used for (b). Also (G) was apparent in other (^{35}S) sulphate treatments of Experiment 21 (Figures 4-21i(b), 4-21ii(a)), so it was most likely that the metabolism of the roots had altered either as a result of changing culture conditions or of a fluctuation of the root's metabolism.

The spots appearing in Experiment 22 [(^{35}S) sulphate 1 hr.] treatment (Fig. 4-19 (c)) contained a considerable quantity of radioactivity. The killing of the roots was different in this experiment; 10 ml. of MCP containing 25 mg. of iodoacetamide being used as an initial extractant instead of 6 ml. MCP containing 20 mg. iodoacetamide. Also, after removing the roots from the radioisotope solution, they were not blotted and weighed as in the other experiments but only blotted, so that the time after removal from the solution to the killing was much less.

If the killing procedure did produce these new spots, then they might represent labile compounds which either reacted more rapidly with the greater amount of iodoacetamide or which otherwise might have disappeared rapidly when the roots were exposed to air during weighing.

The washing of the cation column with MCW before eluting the NF was unlikely to have brought about the production of these compounds as the extract had already been exposed to MCW, although the strong acid resin might have catalyzed a reaction in MCW which does not occur in aqueous media.

If the spots were not the result of a more efficient killing procedure then it appears that the sulphur metabolism of the roots had changed quite markedly.

The appearance of these spots, although complicating the interpretation did not invalidate the comparison of extracts prepared from treatments of any one experiment as all these contained the appropriate new spots that the (^{35}S) sulphate treatments already discussed did (except for the (^{35}S) methionine treatments in Experiment 21, which may be explained from the small quantity of (^{35}S) methionine label transfered.

Experiment 20

The formation of artifacts and the choice of a time to incubate the roots with (^{35}S) sulphate or (^{35}S) methionine

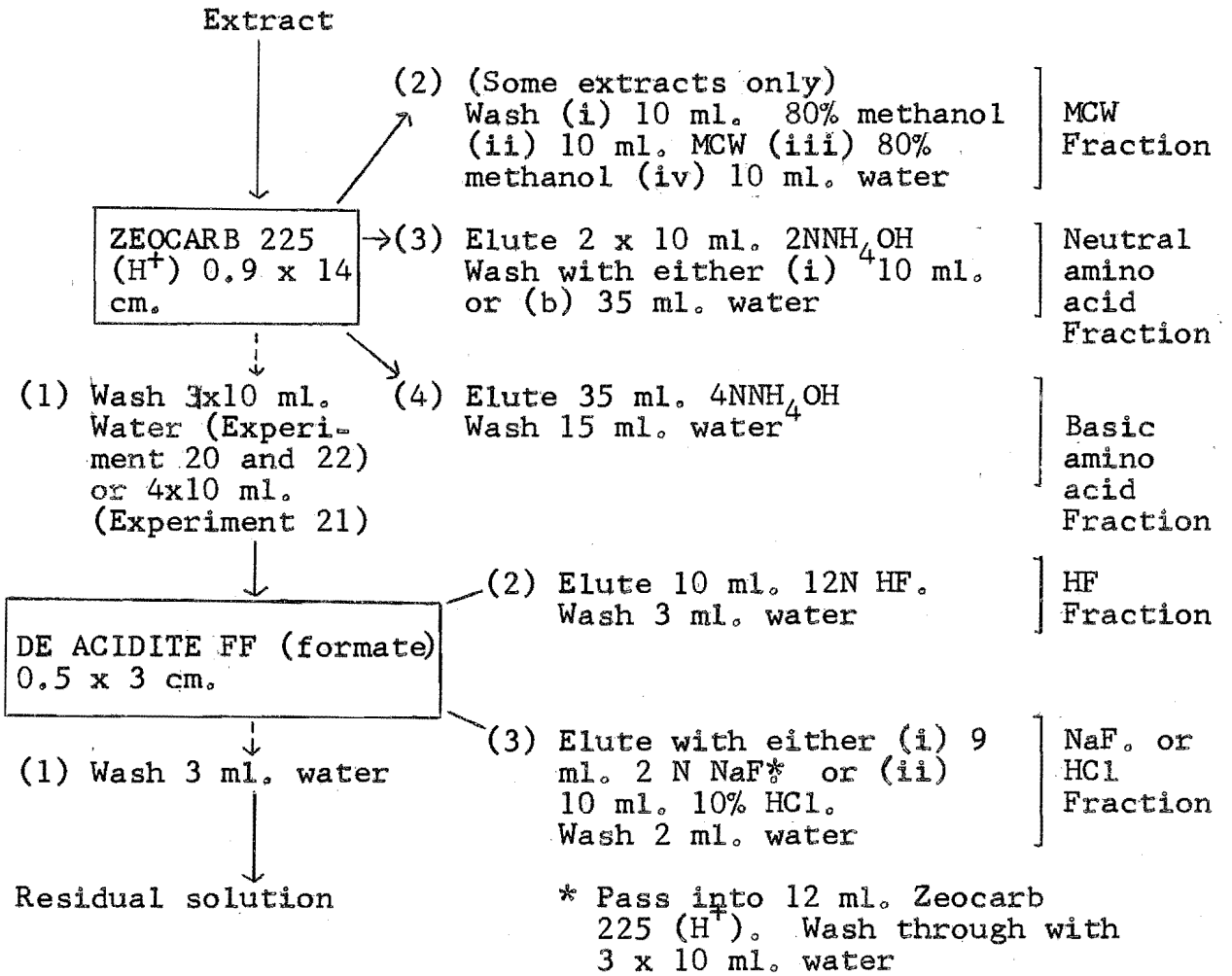
Since Experiment 18 indicated that a one hour incubation of tomato roots at 26°C. with (^{35}S) sulphate was sufficient to label several compounds, this period was again used to establish whether acetamido-cysteine was present in extracts of roots killed with MCP and iodoacetamide.

(^{35}S) methionine was supplied to the roots for the same period of one hour and the extract examined for labelled compounds. Since sulphate was metabolized so extensively in this time, it was expected that methionine would also label cysteine as well as other compounds which might be intermediates in the conversion of methionine to cysteine. In this experiment it was established whether one hour was long enough to label other compounds sufficiently to detect them.

The possible formation of artifacts from (^{35}S) sulphate and (^{35}S) methionine was examined by incubation of these compounds with autoclaved roots in medium and by adding them to an extract of roots, shortly after these had been killed. The former method exposed the labelled molecules to constituents of the incubation medium but the soluble constituents of the roots were also present and the quantity of radioactivity removed with the roots was rather small. On the other hand adding the isotope to an extract did not test for degradation during incubation, although it checked for the production of artifacts during the separation procedures.

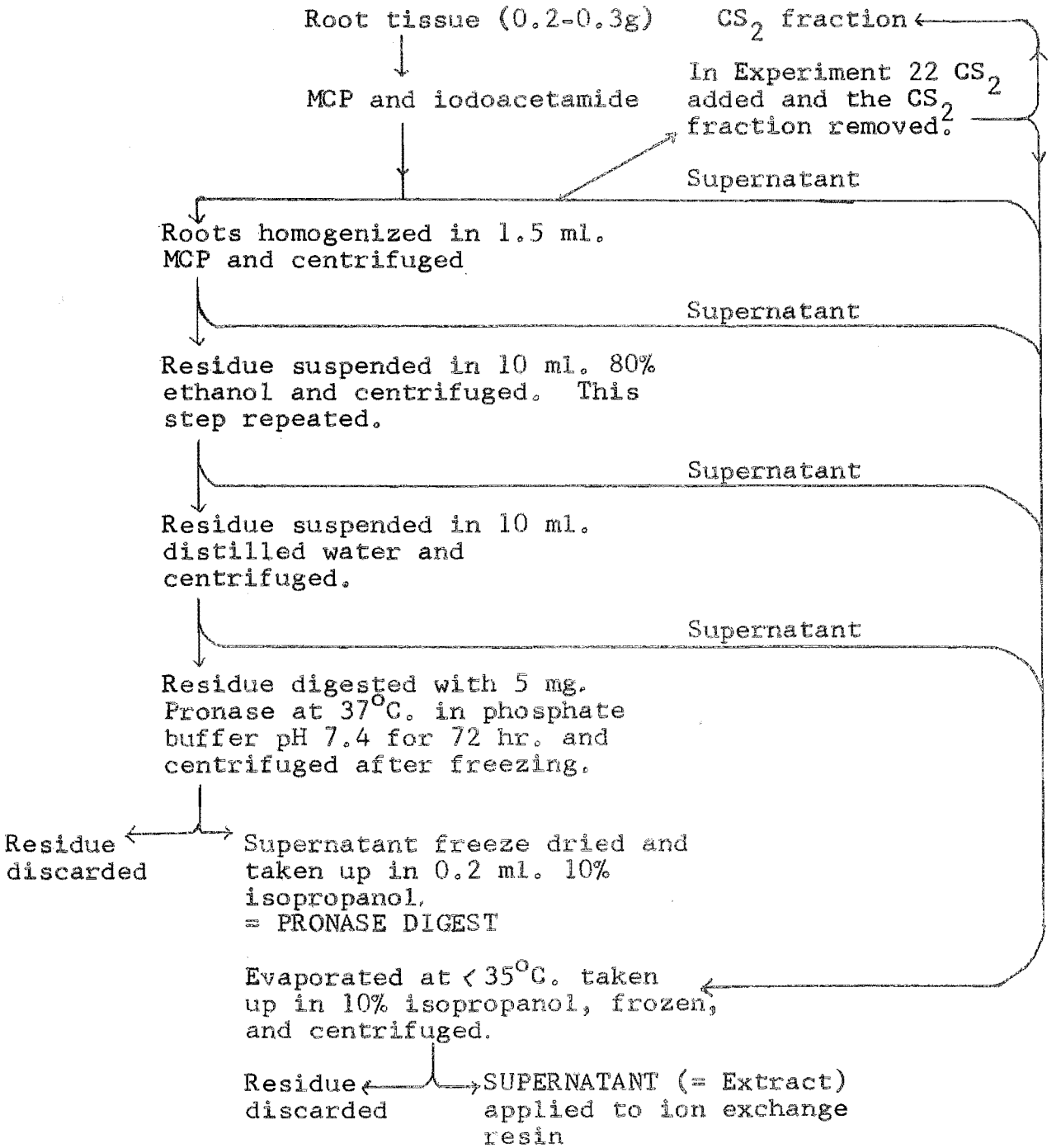
This method did not disclose all the artifacts likely to be found in an extract since only those compounds formed from sulphate and methionine would be present. However the quantity of radioactive sulphate or methionine added was very much in excess of any one of the compounds formed from them so that artifacts from these sulphur sources would be much more prominent in comparison with those from the

Figure 4-20i (b)



Ion exchange fractionation of extracts for Experiments 19-22.

Figure 4-20i (a)



The killing and extracting of tomato roots. In Experiments 19-22.

other metabolites.

Experimental details

I Those in common with Experiments 21 and 22

Each treatment had 11 or 12 seven-day old tomato roots weighing on the average 20-25 mg. fresh weight and grown from tips in 'high magnesium' medium which contained ion exchanged sucrose with either sulphate or methionine. The roots were grown with 16 μg. sulphate-S/25 ml. for this experiment, and with 8 μg. sulphate-S/25 ml. in Experiments 21 and 22. For an experiment, 11 or 12 roots were transfered to 25 ml. of sterile 'high magnesium' medium containing ion exchanged sucrose either without any sulphur compounds or as in Experiment 21, with sulphate or methionine. The medium and roots were contained in a 250 ml. beaker covered with aluminium foil. To a 25 ml. portion of medium was added the appropriate radioisotope; either (^{35}S) sulphate (50 μC.), or (^{35}S) methionine or (^{35}S), all added without carrier. (^{35}S) sulphate solution was autoclaved before addition to the medium. The roots were incubated at 26^{o}C. for the stated time.

The same batch of ion exchanged sucrose which was used in Experiment 16 was used throughout, being kept frozen at less than -15^{o}C. From this experiment the sucrose contained little if any sulphate impurity. The sulphate impurity was reduced to a minimum in order to ensure the greatest uptake of tracer sulphate and so label the sulphur compounds to the greatest extent.

After incubation of the roots with the radioisotope, they were killed and extracted as described in Methods and Materials (3-10). The extract was fractionated by ion exchange and the residue of root material digested with pronase (Methods and Materials - 3-11). In Figure 4-20i (a) is shown a flow diagram of the killing and extraction procedure, while in Figure 4-20i (b) is presented the ion exchange fractionation of the extracts. All the modfications made to the techniques in Experiments 21 and 22 are included in these figures.

The separation of compounds

The NF's of Experiments 20 and 21 were first separated by electrophoresis at pH 5.3 followed by chromatography with PW on 11.5 x 11.5 cm. TL plates. This was found satisfactory for the NF of a tomato root extract in Experiment 18. However the compounds of most interest i.e. methionine, methionine sulphoxide, acetamido-homocysteine, cystathionine and acetamido-cysteine were not separated by PW chromatography after remaining at the origin during electrophoresis. Since these compounds migrated away from the origin at pH 2.0, some of the NF's were then separated by pH 2.0/PW on 11.5 x 11.5 cm. plates. Although several positively charged compounds could be distinguished, including methionine, methionine sulphoxide and acetamido-cysteine, their separation required the whole width of a small plate and the negative compounds were lost. To retain most of the sulphur compounds the extracts were then separated on 20 x 20 cm. plates. At the same time the length of the band spotted on the TL was increased to improve the separation, after which the compounds streaked considerably and a residue of radioactivity was left where the front of the 1% acetic acid, which was used to concentrate the bands into spots, had reached and then been dried. Washing the TL with acetic acid and water (Methods and Materials - 3-13) was found to greatly reduce the streaking, so all the NF's were separated again at pH 2.0/PW and the autoradiograms shown in the figures are from these.

The anion containing fractions were separated at pH 5.3/PW since the anions moved away from the origin at a neutral pH.

For the pH 2.0/PW separations on the 20 x 20 cm. TL plates of the NF's and the one basic fraction (BF), between 4 and 8 μl. of a total volume of 100 μl. for each fraction was loaded on as a band. This represented about 40,000 to 70,000 disintegrations per min. (dpm.). For the HF. and HCl fractions 3 μl. of a total volume of 200 μl. for each fraction was spotted on to a 11.5 x 11.5 cm. plate.

After a separation of the pronase digest from a residue (Figure 4-20iii(b)) at pH 2.0/PW, the other digests were chromatographed in PW on a 20 x 20 cm. plate. Only about 1-2 µl. of the 200 µl. total volume from the pronase digest were spotted on to an origin, since considerable quantities of a brown viscous substance were present in the solution.

The autoradiograms of all TL plates are entire although reduced in size. Where the solvent did not reach the top of the TL, the front is drawn in.

II Experimental details unique to this Experiment

Two treatments of twelve roots in 25 ml. of medium were autoclaved for 10 min. at 10 lbs. before adding the radioisotope.

(^{35}S) methionine was purified by descending chromatography in MPW, followed by ion exchange purification (Methods and Materials 3-16). The methionine contained a radioactive compound besides methionine and MSO. It was unnecessary to sterilize the methionine since the chromatography in MPW would have killed any bacteria or fungi present and the isotope was used immediately after purification.

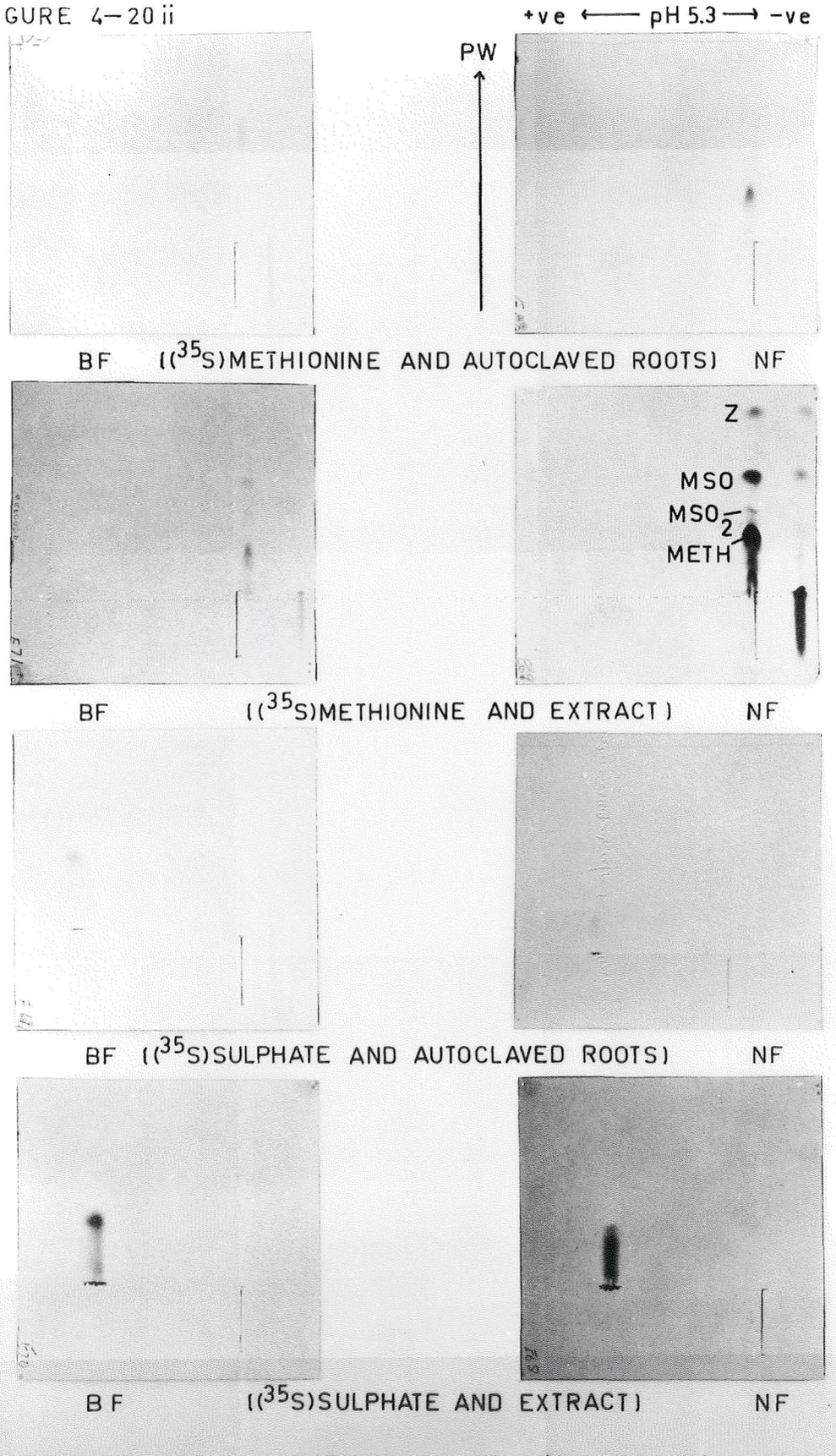
After the radioisotope had been added to the medium, the roots were incubated for one hour. At the end of this time, they were removed with a glass rod, blotted with filter paper, weighed, and placed in MCP with iodoacetamide, as described in Methods and Materials, 3-10.

The roots were homogenized, extracted and the extracts fractionated by ion exchange (Methods and Materials - 3-10, 3-11) into the BF, NF, HF fraction and NaF. fraction.

Separation of compounds

The BF, NF and HF fraction were separated on 11.5 x 11.5 cm. TL plates by electrophoresis at pH 5.3 (500V, 18 min.) followed by chromatography in PW. In addition the NF of the [(^{35}S) sulphate and autoclaved roots] extract was separated on a 20 x 20 cm. TL plate (1,140 V, 14 min.) and the NF of the [(^{35}S) sulphate one hour] treatment was

FIGURE 4—20 ii



EXPERIMENT 20. AUTORADIOGRAMS OF NF'S AND BF'S FROM TOMATO ROOT EXTRACTS.

separated at pH 2.0/PW (1,140 V, 40 min.). The separations on the small plates were not repeated for the others since the compounds were adequately distinguished.

Treatments

[(^{35}S) sulphate one hour]: roots incubated one hour.

[(^{35}S) sulphate and autoclaved roots]: autoclaved roots incubated one hour.

[(^{35}S) sulphate with extract]: radio-isotope added to extract 3 min. after the roots.

[(^{35}S) methionine one hour]: roots incubated one hour.

[(^{35}S) methionine and autoclaved roots]: as for sulphate.

[(^{35}S) methionine with extract]: as for sulphate.

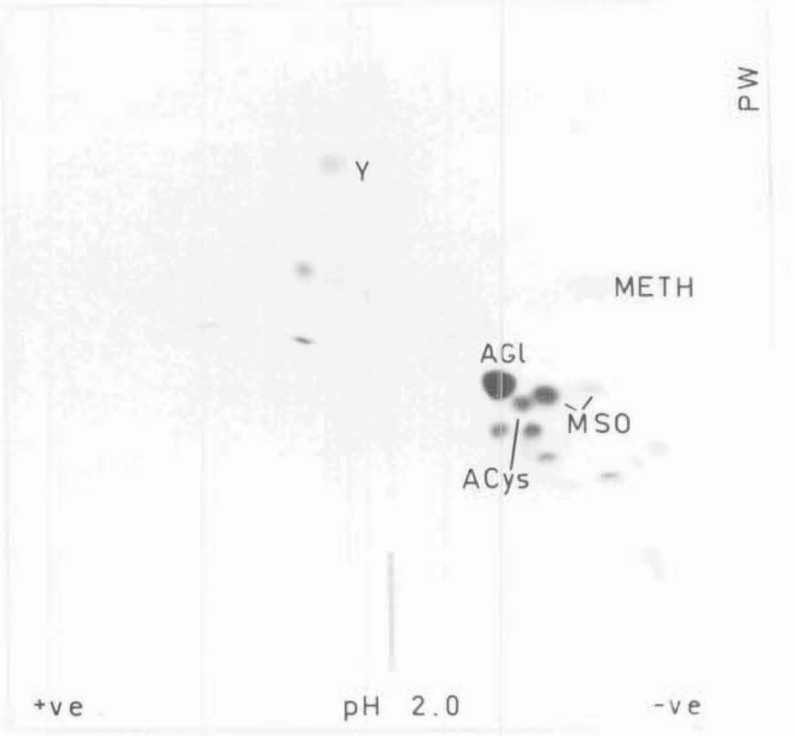
Results and Discussion

In Figure 4-20ii are shown autoradiograms of the BF's and NF's of the extracts and autoclaved roots. The single radioactive spot apparent in the NF and BF of the [(^{35}S) sulphate and extract] treatment was considered to be sulphate from its position. (^{35}S) sulphate was also present in the NF and BF of autoclaved roots, along with a slight negatively charged spot and a positive one. A positively charged compound similar to the latter also appeared in the [(^{35}S) methionine with extract] treatment. Since methionine and its oxidation products appeared to be duplicated above this positive compound, it was considered that this was in part a complex, which released the amino acids during chromatography. That a similar compound was present in the sulphate treatment supported this.

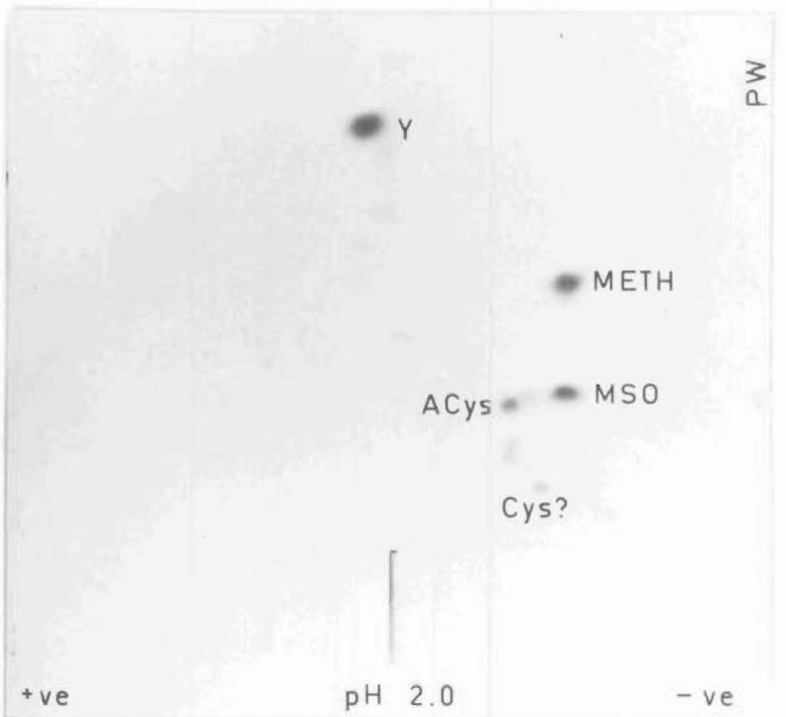
In the NF of the [(^{35}S) methionine and extract] treatment were present spots corresponding to methionine, methionine sulphoxide (MSO) and methionine sulphone (MSO_2) as well as another with a higher Rf than methionine (Z). The significance of Z was unknown since it was not apparent in the later experiments with (^{35}S) methionine.

Some methionine was present in the BF also. This could have been the result of insufficient washing of the column after elution with 2N ammonia. The extra compound present in the methionine supplied to the roots was most

FIGURE 4–20 iii



(a) NF OF THE EXTRACT



(b) PRONASE DIGEST

EXPERIMENT 20. AUTORADIOGRAMS OF THE NF AND THE PRONASE DIGEST OF ROOTS INCUBATED WITH (^{35}S)SULPHATE 1 HR..

likely to be coincident with methionine since basic solvents such as MPW and Pyr.W failed to separate them and BAW did, or else was equivalent to Z.

The presence of (^{35}S) sulphate in the BF could not be explained by insufficient washing of the column. The passage of sulphate through the column should have been rapid since being negative it is excluded from cation resin beads. Because sulphate seemed to form a positive complex in the extract it was possible that this was retained by the ion exchange resin.

No spots appeared on the autoradiogram of the [(^{35}S) methionine with extract] HF. fraction.

The HF. fraction of the [(^{35}S) sulphate with extract] treatment contained one radioactive compound (Appendix Figure 1). The HF. fraction was not further considered since in the later experiments with roots radioactive compounds were not prominent in it, nor were they present at shorter exposure periods than one hour or in (^{35}S) methionine fed roots] (Appendix Figure 1).

The radioactive compounds formed by roots from (^{35}S) sulphate

Figure 4-20iii (a and b) show autoradiograms of a pH 2.0/PW TL separation of the NF and of the pronase digest from [(^{35}S) sulphate one hour]. In (b), the spot labelled Cys was considered to be cystine from its position and the correspondence of a ninhydrin spot with it. After one hour several compounds of the extract were intensely labelled, including ACys ((Y) was a degradation product of ACys.) The protein fraction contained both labelled cysteine and methionine.

The radioactive compounds formed by roots from (^{35}S) methionine

In a pH 5.3/separation of the NF, it was found that apart from several positive compounds, similar to those in the [(^{35}S) methionine and extract] treatment and the oxidation products expected from methionine, a slight amount of radioactivity was present at the position of ACys and AG1. Although the spots were rather weak, this incubation period was continued since sulphate labelled many compounds

in this time and a comparison with it was made later. The metabolism of methionine in this experiment was not further discussed because of the presence of an impurity in the (^{35}S) methionine solution fed to the roots and because this treatment was repeated in Experiment 21.

Experiment 21

(a) The affect of shorter time on labelling of compounds from (^{35}S) sulphate

Since in Experiment 20 the number of compounds labelled after one hour was quite large, the time of incubation with (^{35}S) sulphate was reduced in this experiment to emphasize those formed more directly from sulphate. As well as an incubation period of one hour, which was also a part of the second half of this experiment, incubation periods of half and quarter hours were used. For these last two a greater quantity of extract was spotted on the TL plates to compensate for the reduced quantity of radioactivity, so that the intensity of the major spots remained more or less constant.

(b) The metabolism of (^{35}S) methionine

The metabolism of (^{35}S) methionine was investigated to establish how the roots in Experiment 13 produced cysteine from methionine for protein synthesis. The HCl fraction as well as the NF was examined to establish whether cysteine might be formed from sulphate after the latter had been produced from methionine.

As the fresh weight and dry weight gain of roots supplied with methionine was greater than that when they were supplied with sulphate at the lower concentrations in Experiment 13 and the normal sulphur source for plants is sulphate it was possible that methionine altered the metabolism of sulphur compounds in tomato. Davies (1966) had reported that exogenous methionine regulated the respiration rate of washed turnip discs. To detect any such change, (^{35}S) methionine and (^{35}S) sulphate were fed to roots grown on methionine as well as to roots grown on sulphate. (^{35}S) sulphate was fed in case only the compounds formed from methionine and cysteine were labelled by the former, as was indicated by Experiment 20, and any change in metabolism was restricted to the compounds formed from sulphate.

Design

[(^{35}S) sulphate 1/4 hr.]: roots incubated with sulphate 1/4 hr.

[(^{35}S) sulphate 1/2 hr.]: roots incubated for 1/2 hr.

[(^{35}S) sulphate alone]: roots incubated for 1 hr.

[(^{35}S) sulphate and methionine]: roots were grown with methionine and incubated with both (^{35}S) sulphate and methionine for 1 hr.

[(^{35}S) methionine]: roots grown with methionine and incubated with (^{35}S) methionine for one hour.

[(^{35}S) methionine and sulphate]: roots grown with sulphate and incubated with both (^{35}S) methionine and sulphate for 1 hr.

Experimental details

Since these concentrations gave optimum growth and were not inhibitory, tomato roots were grown either with 8 µg. sulphate-S/25 ml. or with 3 µg. methionine-S/25 ml. Where (^{32}S) sulphate or (^{32}S) methionine was present with the radioisiotope, the same concentrations were used.

(^{35}S) methionine was purified before ion exchange separation by descending chromatography in both BAW and MPW in separate runs. The radioactivity was determined from a small portion of the purified solution dried on a planchet and 8 µC. of (^{35}S) methionine was added to the 25 ml. of medium. From the specific activity stated by Amersham, this contained 2.2 µg. methionine so that it would not have been inhibitory.

The killing, homogenizing and extraction of the roots was carried out as described for Experiment 20, but the procedure for ion exchange fractionation was slightly different from that in Experiment 20. To prevent (^{35}S) sulphate from appearing in the NF the cation colum was washed with 40 instead of 30 ml. boiled water before elution with ammonia and the washing after elution with 2N ammonia was with 35 ml. instead of 10 ml. to prevent the appearance of methionine in the BF.

The strongly basic fraction (NaF. fraction) was eluted from the anion resin with sodium formate (NaF.) for the [(^{35}S) sulphate] treatments but some residual NaF. was sometimes present after ion exchange. For the [(^{35}S) methionine] treatments, HCl was used to elute this fraction (HCl fraction).

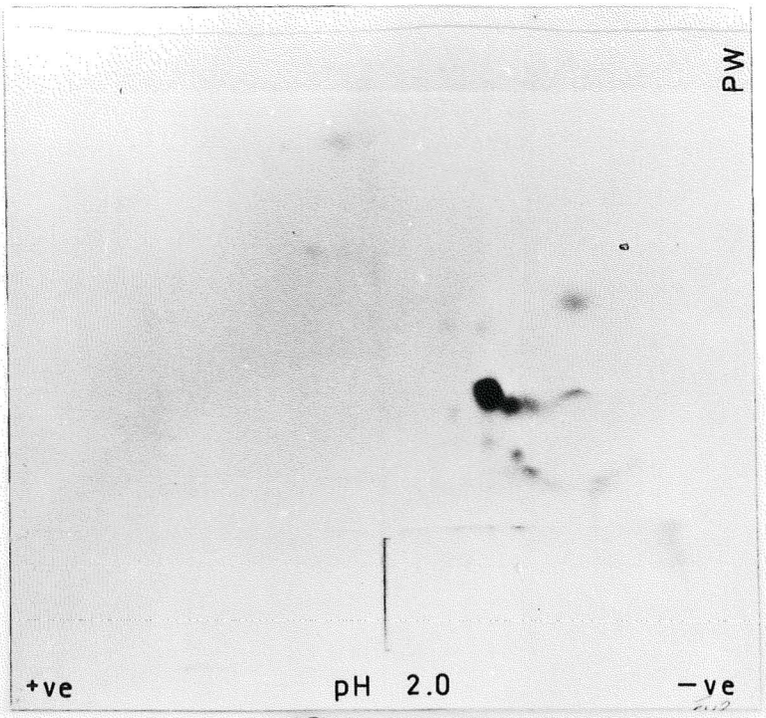
After separation of the compounds, the TL plates were exposed to X-ray film for 8 days.

Results and Discussion

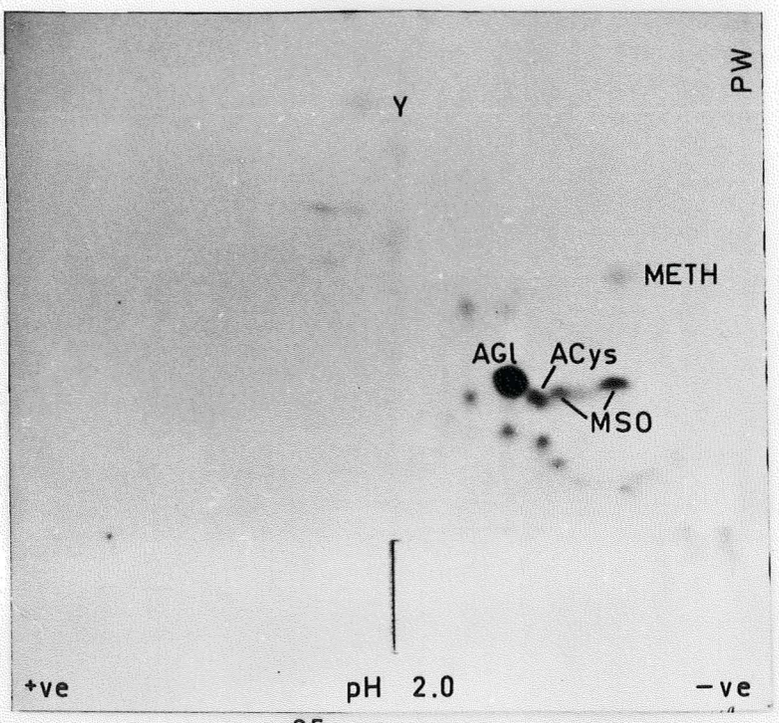
The fractionation of the extract

Although the quantity of water used to rinse the cation column after 2 N ammonia elution was increased from 10 to 35 ml. the BF still contained 13% of the radioactivity in the NF. Since the same amino acids, with two exceptions, were present in the BF as in the NF (for example see Figure 4-22ii), this slow elution cannot be explained entirely by the presence of more basic compounds in the BF. It was most likely that the interior of the beads was not converted entirely to the ammonium form and so some of the neutral amino acids were retained. At the low temperature - less than 5°C. - diffusion rates are slower than at room temperature, and the mesh size of the beads was 52-100 which was much larger than the 200-400 used by Thompson et al. (1959).

FIGURE 4—21 i



(a) ((35S)SULPHATE 1/4 HR.)



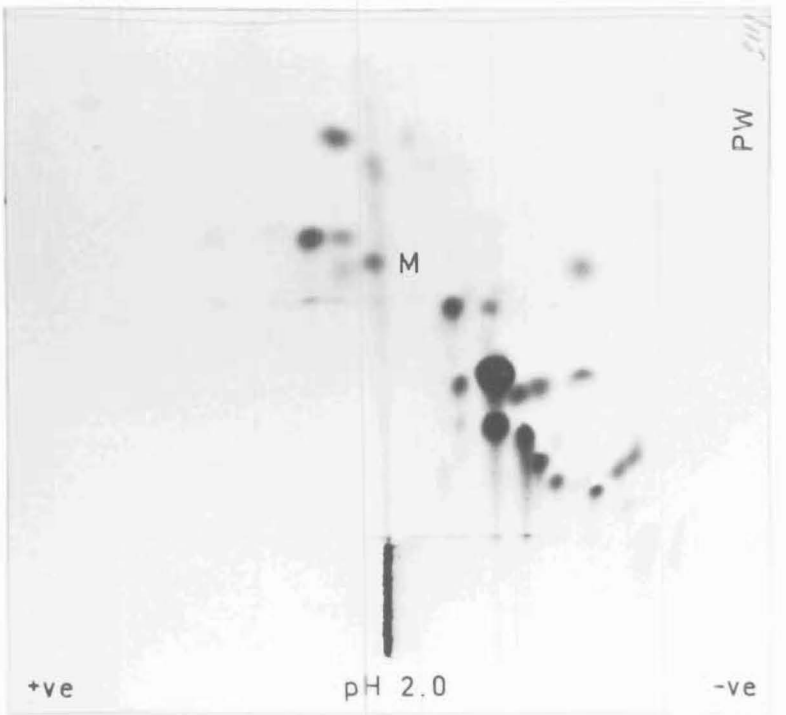
(b) ((35S)SULPHATE 1/2 HR.)

EXPERIMENT 21. AUTORADIOGRAMS OF NF'S FROM TOMATO ROOT EXTRACTS.

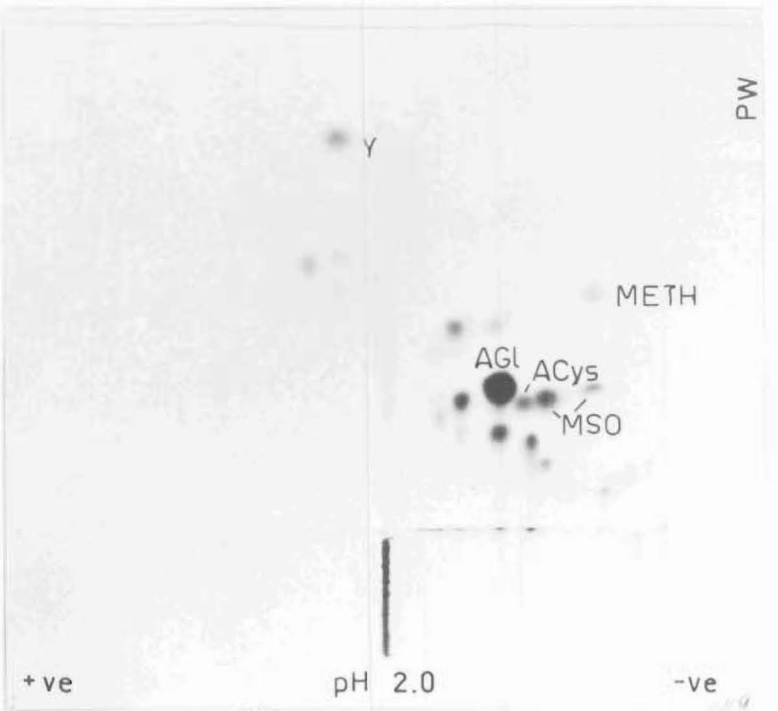
(a) The affect of a shorter incubation time on the labelling of sulphur compounds

In Figure 4-21i (a and b) are shown autoradiograms of pH 2.0/PW separations of the NF's from extracts of tomato roots. The intensity of the AG1 spot was already the highest after a quarter-hour and that of the ACys (plus Y) and methionine (plus MSO and MSO_2) was marked. Since Y, MSO and MSO_2 are formed during extraction and separation, these are included with the parent compounds. Several of the other spots were visible. This indicated that the flow of radioisotope was sufficiently rapid to extensively label the sulphur compounds after one hour. From a comparison of the intensity of the spots on the 1 and 1/2 hr. autoradiograms the increase of radioactivity after 1/2 hr. was similar for all of the spots. Hence equilibrium of the flow of radioisotope between the compounds was reached after an hour or so. This was supported by the similar ratios of radioactivity in protein ACys (plus Y) to that in protein methionine present in the pronase digests for the three incubation times (Figure 4-21iv). These patterns show that glutathione, cysteine and methionine are important metabolites in sulphate metabolism but their sequence was not established.

FIGURE 4–21ii



(b) ((35S)SULPHATE AND METHIONINE)



(a) ((35S)SULPHATE ALONE)

EXPERIMENT 21. AUTORADIOGRAMS OF NF'S FROM TOMATO ROOT EXTRACTS. ROOTS INCUBATED WITH (^{35}S)SULPHATE 1 HR..

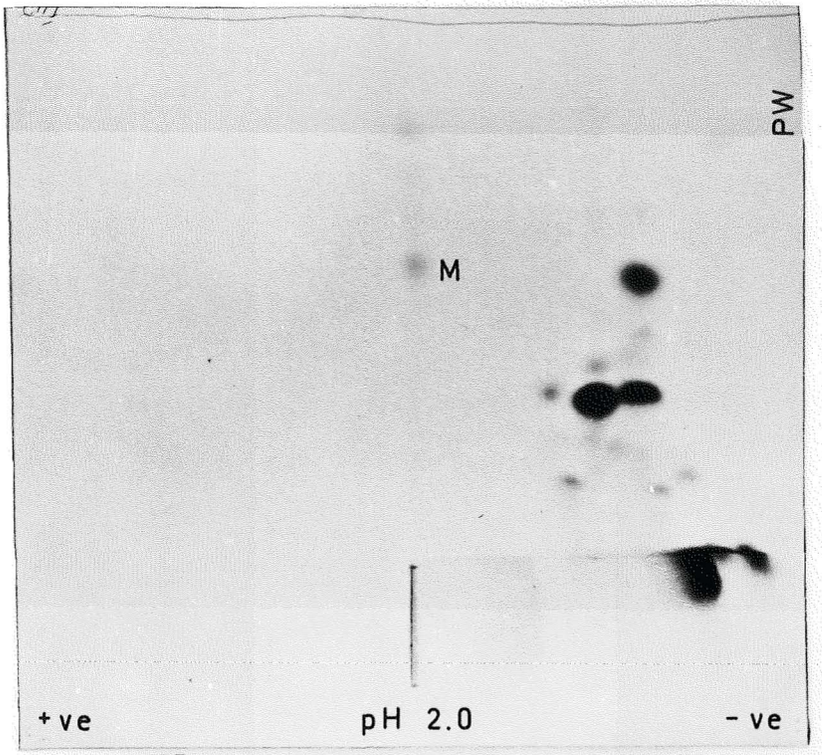
(b)i The metabolism of (^{35}S) sulphate as affected by methionine

In Figure 4-2iii are shown autoradiograms of pH 2.0/PW separations of the NF's of extracts of tomato roots treated as described. If allowance is made for the increased amount of radioactivity in the [(^{35}S) sulphate and methionine] treatment, the overall patterns were similar. The labelling of methionine especially seemed to be unaffected, but Figure 4-2iv shows that protein methionine was scarcely labelled by the (^{35}S) sulphate. The labelling of the soluble pool but not the protein methionine can be explained by either two soluble methionine pools existing, or by the soluble methionine pool greatly increasing in size, so improving the efficiency of detection, or by the soluble methionine becoming labelled at the end of the incubation period. Since excess methionine was provided this last alternative was unlikely. Either of the other alternatives means that little methionine was synthesized from sulphate compared with that in sulphate-alone fed roots.

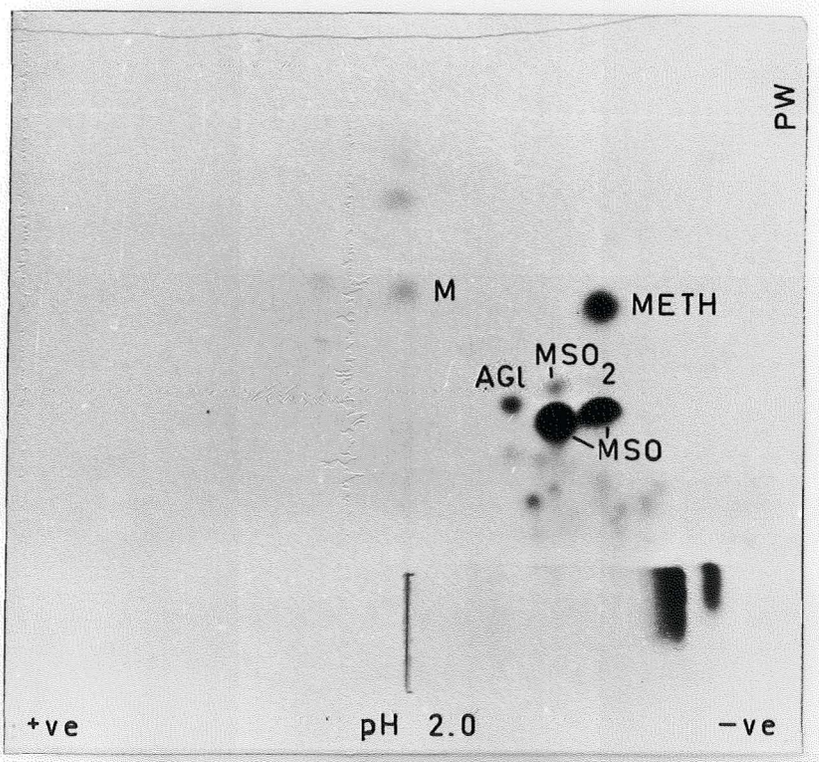
The most noticeable difference between the two treatments was the increased intensity of spot M in the methionine fed treatment. A faint spot in this position was apparent in the other NF's of the [(^{35}S) sulphate 1 hr.] treatments and was slightly more intense than this in the (^{35}S) methionine fed roots (Figure 4-2iii). Since few compounds were labelled by methionine this one might have been closely related to methionine and perhaps lies between cysteine and methionine.

However, the metabolism of (^{35}S) sulphate was hardly affected by growing the roots on methionine, so that the metabolism of (^{35}S) methionine was not expected to be abnormal.

FIGURE 4–21 iii



([^{35}S]METHIONINE AND SULPHATE)



([^{35}S]METHIONINE ALONE)

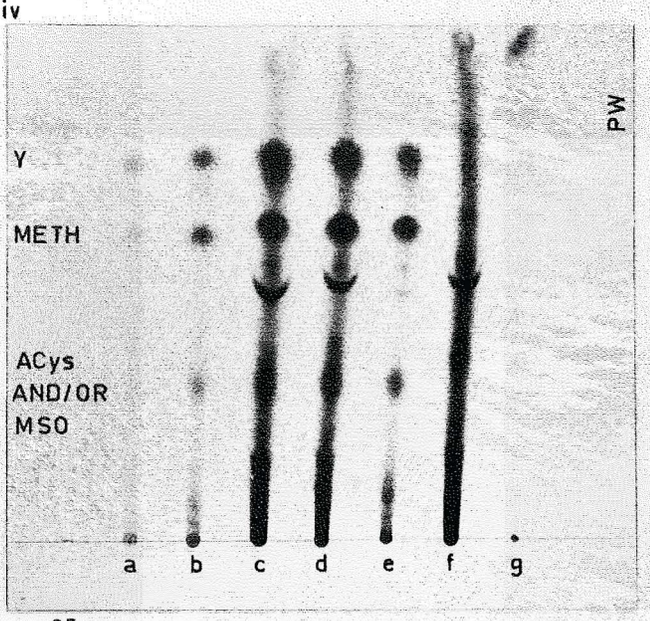
EXPERIMENT 21. AUTORADIOGRAMS OF NF'S FROM TOMATO ROOT EXTRACTS. ROOTS INCUBATED WITH ([^{35}S]METHIONINE FOR 1 HR..

(b)ii The compounds formed from (^{35}S) methionine and the affect of growing the roots with methionine on (^{35}S) methionine metabolism.

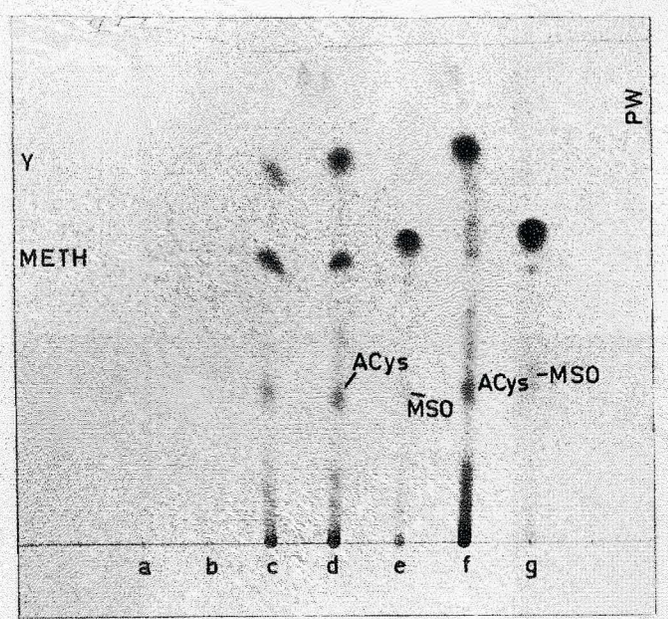
In Figure 4-21iii are shown autoradiograms of pH 2.0/PW separations of the NF's of extracts from tomato roots incubated with (^{35}S) methionine. Apart from methionine (and MSO and MSO_2) the only other identified compound was AG1. No ACys (or breakdown product Y) was noted nor could a spot be detected at the positions of cystathionine or S-methyl-cysteine (from the standard map). At the same time methionine in protein (Figure 4-21iv) was labelled but not cysteine, regardless of whether sulphate was added to the medium.

Since AG1 was labelled and glutathione is formed from cysteine (Young and Maw, 1958), cysteine was synthesized from methionine. In E.coli the cysteine in glutathione was also derived rapidly from the soluble cysteine (Roberts et al. 1955). Since on the one hand cysteine was labelled when (^{35}S) sulphate alone was fed and on the other it was not labelled when (^{35}S) methionine and (^{32}S) sulphate were fed, it was formed almost exclusively from sulphate. If it is assumed that the fresh weight increased logarithmically with time, then in an hour the 11 or 12 roots would have grown about 11 mg.. The amount of sulphate present in carrier radiosulphate was insufficient for this amount of growth so that either a sulphur impurity or an internal sulphur source contributed to the synthesis of cysteine. From Experiment 16, 25 ml. of medium made with this batch of ion exchanged sucrose contained sufficient sulphate impurity for 4.7 mg. fresh weight gain. Since protein cysteine and methione were more or less equally labelled when (^{35}S) sulphate alone was fed to the roots, this impurity would have been sufficient to synthesize most of the cysteine when methionine was fed as well. The sulphate present would have been greater in the [(^{35}S) sulphate] treatment since these were grown with sulphate and so would have contained free space sulphate as well as internal sulphate. The tomato roots therefore synthesized cysteine from sulphate in preference

FIGURE 4-21iv



EXPERIMENT 21.[(^{35}S)SULPHATE] TREATMENTS;(a)1/4 HR.; (b)1/2 HR..
EXPERIMENT 22:[^{35}S] TREATMENTS;(c)1/2 HR.; (d)1 HR.: [(^{35}S)SULPHATE 1 HR.](e): (f)[(^{35}S 1 HR.] CARBON DISULPHIDE FRACTION. (g) ^{35}S STANDARD.



EXPERIMENT 20:(a)[(^{35}S)METHIONINE AND EXTRACT], (b)[(^{35}S)SULPHATE AND EXTRACT], (c) [(^{35}S)SULPHATE 1 HR.].
EXPERIMENT 21: (d)[(^{35}S)SULPHATE ALONE], (e)[(^{35}S)METHIONINE ALONE], (f)[(^{35}S)SULPHATE AND METHIONINE], (g)[(^{35}S)METHIONINE AND SULPHATE].

AUTORADIOGRAMS OF PRONASE DIGESTS FROM TOMATO ROOTS FOR EXPERIMENTS 20–22. ACys AND MSO WERE IDENTIFIED BY ELUTION AND ELECTROPHORESIS AT pH 2.0 ALONGSIDE STANDARDS.

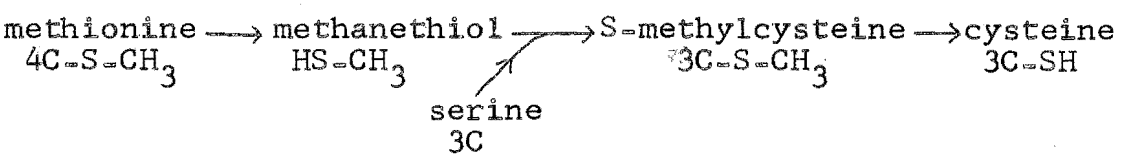
to methionine even though the former was present in smaller quantity than was methionine, but they incorporated exogenous methionine into protein in preference to methionine synthesized from sulphate.

Although little glutathione was formed from (^{35}S) methionine the HCl fraction of [(^{35}S) methionine] treatment contained a total of 400 cpm. and that from [(^{35}S) methionine with sulphate] contained 200 cpm. (The counting efficiency for these figures was 6.3%). After a separation at pH 5.3/ PW of a tenth of the latter, no spots were visible on the autoradiogram and several spots were present on the autoradiogram of a similar separation of 1.5% of the HCl fraction (Figure 4-22iii) from the (^{35}S) sulphate treatment of Experiment 22.

In Experiment 22 [(^{35}S) sulphate] treatment the radioactivity in the HCl fraction was seven times that in the NF (Table 4-22) and most of the former was in sulphate (Figure 4-22ii). The [(^{35}S) sulphate] treatment of this experiment was not used for comparison because of the large amounts of sodium formate still present in the NaF. fraction which would reduce the counting efficiency. Some of the (^{35}S) sulphate found in this fraction would have been present in the free space but since 40% of the radioactivity added in Experiment 22 was removed in the roots and the major fraction of this was (^{35}S) sulphate the proportion would have been less than 5% (assuming root free space = 100%). Since in this experiment the amount of radioactivity in all the compounds increased proportionately after half an hour, the amount of radioactivity in (^{35}S) sulphate needed to label AG1 after one hour would have been easily visible because it should have been seven times greater than that present in AG1. That no (^{35}S) sulphate was visible suggested that cysteine was not synthesized from sulphate derived from methionine.

Although neither cystathionine nor homocysteine - the two intermediates in the fungal conversion of methionine to cysteine - was located in extracts, the formation of cysteine

from methionine was unlikely to be through sulphate. The pathway suggested from the results of Sugii et al. (1963)



also seemed unlikely since no S-methylcysteine or its sulphoxide seemed to be present either in (^{35}S) sulphate or (^{35}S) methionine fed roots. Also Thompson and Gering (1966) concluded that in radish S-methylcysteine was formed from methionine through cysteine.

The failure to find homocysteine (as AHCys) and cystathionine does not rule out conversion of methionine into cysteine through them since the reverse is the most likely pathway for the synthesis of methionine and so they should have been present. It is conceivable that AHCys broke down more completely than did ACys and the degradation product alone was present.

From these results it is most likely that methionine was transformed into cysteine through homocysteine and cystathionine but the proof of this must await the identification of these two compounds after (^{35}S) methionine feeding. This should be easily accomplished when methionine is a sole source of sulphur and no sulphate is present.

Experiment 22

(a) To test elemental sulphur for the presence of oxidized sulphur compounds

Mellor (1930) stated (p. 88) in a summary of the literature that elemental sulphur was oxidized in water or in damp air to sulphate. Although radioactive elemental sulphur was dissolved in benzene, its rate of oxidation might have been increased by radiolysis of the sulphur. The presence of sulphate and some other sulphur anions in elemental sulphur would have made labelling of sulphate or the amino acids in the plant difficult to interpret, since their label is transferred in horseradish, through methionine to Sinigrin - a thioglucoside, (Wetter, 1964; Chisholm and Wetter, 1964). The solution of elemental sulphur was therefore examined for the presence of anions.

If there were any sulphur anions present these would be removed by washing a solution of sulphur in an organic solvent with water, since elemental sulphur is insoluble in water (Mellor, 1930). The (^{35}S) elemental sulphur supplied in benzene was dried at room temperature and dissolved in chloroform as the latter is heavier than water and so could be removed after extraction by running out of the bottom of a separatory funnel.

Experimental details

From a solution of (^{35}S) elemental sulphur in chloroform, fifty µl. containing about 30 µC. was added to 3 ml. of chloroform in a 250 ml. separatory funnel. To extract the sulphur anions, 10 ml. of double distilled water was added to the funnel and shaken with the chloroform. The latter was then run out and the aqueous solution extracted twice with 2 to 3 ml. of chloroform, once with 25 ml., twice more with 2 to 3 ml. and then with 25 ml.. After the radioactivity in 50 µl. (I) had been determined on a stainless steel planchet, the solution was extracted a further four times with 3 ml. of chloroform, the activity being again determined after the second (II) and fourth (III) extraction. The solution was then finally extracted with 25 ml. of petroleum ether and after it had been run out of

FIGURE 4-22 (a)

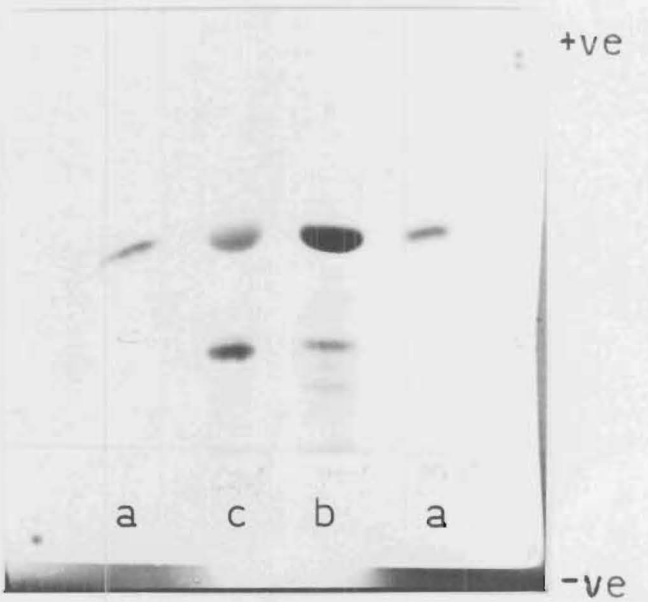


FIGURE 4-22(a). Autoradiogram of the water extract of a ^{35}S solution in chloroform after electrophoresis at pH 5.3. (a) (^{35}S)sulphate standard. (b) Extract. (c) Extract oxidized with 3% hydrogen peroxide.

the separatory funnel 50 µl. of it was taken for counting (IV).

To identify the nature of the radioactive species, the solution was evaporated at less than 40°C., taken up in 0.5 ml. of 10 per cent isopropanol and stored at less than -15°C.. Twenty µl. of this solution was electrophoresed alongside (^{35}S) sulphate at pH 5.3, with and without hydrogen peroxide oxidation.

Results and Discussion

Successive determinations of radioactivity in water extract.		Cpm.
	I	53
	II	44
	III	52
	IV	50

The determination of radioactivity in 50 µl. of a 10 ml. water extract of elemental radioactive sulphur.

The table shows that except for the second determination (II), the specific activity of the solution remained constant within ± 3 per cent of the average during the successive extractions with chloroform and petroleum ether. It was presumed that the second determination was lower from an error in pipetting the solution onto the planchet.

Electrophoresis at pH 5.3 (Figure 22(a)) showed that a compound with the mobility of sulphate as well as others of lesser mobility were present. Some of these compounds were oxidized by the hydrogen peroxide, and so, as well as sulphate, other sulphur anions of lower oxidation state were probably present in the elemental sulphur.

To purify samples of elemental sulphur in chloroform from anions, it would be satisfactory to wash the chloroform with water four or five times since the anions would be far more soluble in water than in the chloroform. In the same way, it would also be possible to determine the amount of oxidation of elemental sulphur in water by extracting it from the water with chloroform and determining

the specific activity of the aqueous solution, since the constant specific activity during extraction with chloroform showed that oxidized sulphur compounds are quantitatively retained in the water.

If it is assumed that the original extraction of the chloroform with water removed mostof the sulphur anions then they were approximately 0.5 per cent of the total sulphur, which although not very great would have been sufficient to label the sulphur compounds in a plant.

Experiment 22

(b) The assimilation of elemental sulphur by tomato roots

Since Experiment 16 showed that the preparation and autoclaving of the colloidal sulphur solution did not produce a water soluble sulphur source for tomato roots, oxidation of the sulphur in water was unlikely, and so (^{35}S) was fed to roots to see whether (^{35}S) sulphate was formed. If there was none in the roots then elemental sulphur would have been assimilated directly into organic compounds, but if there was the sulphur might equally have been oxidized to sulphate in the medium as in the roots. For comparison and as part of Experiment 19, a treatment of (^{35}S) sulphate - fed roots was included.

Experimental details

About 1 mC. of (^{35}S) from a solution of ^{35}S in benzene was evaporated with a stream of N_2 in a tapered centrifuge tube and 0.6 ml. of distilled 'Pronalys' chloroform was added to the residue, followed by 2 ml. of double distilled water and the two liquids were shaken gently before being centrifuged at 1000 g for 5 min.. The water solution containing sulphur anions (as shown in Part (a)) was removed with a 'Transpet' and the surface of the chloroform rinsed with 2 ml. of double distilled water. This extraction was repeated three times and each time one drop was taken to measure the radioactivity in it. At first a drop contained about 13,000 cpm. (uncorrected) and at the end about 10,000 cpm.. This level of radioactivity was too high after four extractions to be just sulphur anions and so some elemental sulphur was present. The slight drop of radioactivity indicated that some of the sulphur anions were extracted.

To prevent oxidation, the chloroform solution with the remaining water was evaporated at 25°C. with a stream of N_2, from a glass capillary and 0.42 ml. of acetone was added. From the solution, 0.1 ml. was transfered to a solution of 5 mg. sulphur in 50 ml. acetone, and the remaining acetone solution transfered to 1.2 ml. of double

distilled water, which was evaporated down to 0.8 ml. to remove the acetone. To each of the treatments containing elemental sulphur was added 0.1 ml. of this colloidal solution and this aliquot contained about 42 μC.

After the ^{35}S had been added to the medium, the roots were incubated for either half an hour or one hour. The [^{35}S with extract] treatment was prepared by adding the roots to the MCP extractant, followed 3 min. later by 0.1 ml. of the colloidal ^{35}S solution. A treatment of roots was incubated with (^{35}S) sulphate for one hour.

The roots were killed in 10 ml. of MCP containing 25 mg. iodoacetamide at 5-10°C. Since elemental sulphur was volatile, the extraction procedure was modified to prevent the radioactivity from being hazardous. To each of the beakers containing killed roots for the elemental sulphur treatments was added 10 mg. flowers of sulphur along with 5 ml. of 'Analar' carbon disulphide so that the ^{35}S was diluted with carrier. Before the roots were homogenized and extracted (Methods and Materials 3-10), a little more CS_2 was added to replace evaporation losses, the roots were transfered to the pestle and the top phase of MCP transfered to the evaporating flask to which the supernatant of each of the extractions was to be added. A little of the MCP was left on top of the CS_2 so both were dried in a stream of air and the elemental sulphur transfered with 5 ml. of carbon disulphide to a vial. A portion was counted.

After the roots had been homogenized and extracted, the extracts were fractionated by ion exchange (Methods and Materials - 3-11) with the modification described in Experiment 21 including a washing of the cation column with methanol: chloroform; water (MCW, same proportions as MCP) before commencing elution of the NF. This was carried out because, of the (^{35}S) sulphate added to an extract, 0.3% was recovered in an MCW eluate of the cation column after first rinsing the resin with 40 ml water. In this experiment the extract was first rinsed through with 30 ml. of boiled double distilled water and then

the cation resin was washed with 10 ml. of 80% methanol, 10 ml. MCW, 10 ml. of 80% methanol and 10 ml. of water. All these washings were bulked (MCW fraction). The NF was then eluted with 2 N ammonia.

In Experiment 21 it was noticed that one of the treatments contained not only much less radioactivity in the NF than expected but also a lesser quantity of the amino acids. Therefore in this experiment the quantity of radioactivity added to the column in the extract and that present in each of the eluted fractions as well as the fraction passing through both columns was estimated from portions pipetted from the eluates onto an aluminium planchet, before they were evaporated on the 'Buchi'. Stainless steel planchets were used for the HCl fractions since these attacked the aluminium. The recovery of radioactivity from the columns had not been measured before because the error in measuring the radioactivity in the large amount of sulphate would obscure a small difference in the recovery of the neutral amino acids. In Experiment 21 such a large decrease of radioactivity from that expected was found that the recovery was estimated in this experiment.

Design

[^{35}S 1/2 hr.]: roots incubated with sulphur for half an hour.
[^{35}S 1 hr.]: roots incubated with sulphur for one hour.
[^{35}S and extract]: ^{35}S added to MCP extractant 3 min. after the roots were.
[(^{35}S) sulphate 1 hr.]: roots incubated with 50 μC. (^{35}S) sulphate for one hour.

Table 4-22

% recovery	Treatment	Radioactivity (cpm x 10^{-6}) Added to column	BF	NF	HF fraction	HCl fraction	MCW	Residue
93	[(^{35}S)sulphate 1 hr.]	0.681	0.011	0.072	0.017	0.476	0.034	0.007
73	[^{35}S 1/2 hr.]	1.540	0.023	0.143	0.053	0.861	0.028	0.016
112	[^{35}S 1 hr.]	1.220	0.040	0.214	0.105	0.974	0.030	0.012
102	[^{35}S and extract]	1.324	0.053	0.287	0.217	0.058	0.274	0.462

Efficiency of counting = 2.35%

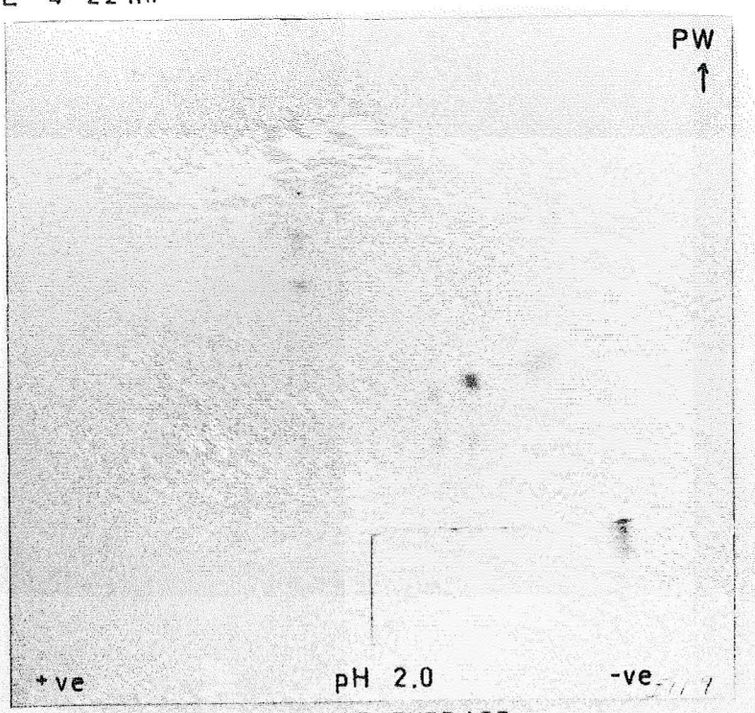
The recovery of radioactivity from the extracts applied to the ion exchange columns.

Results and Discussion

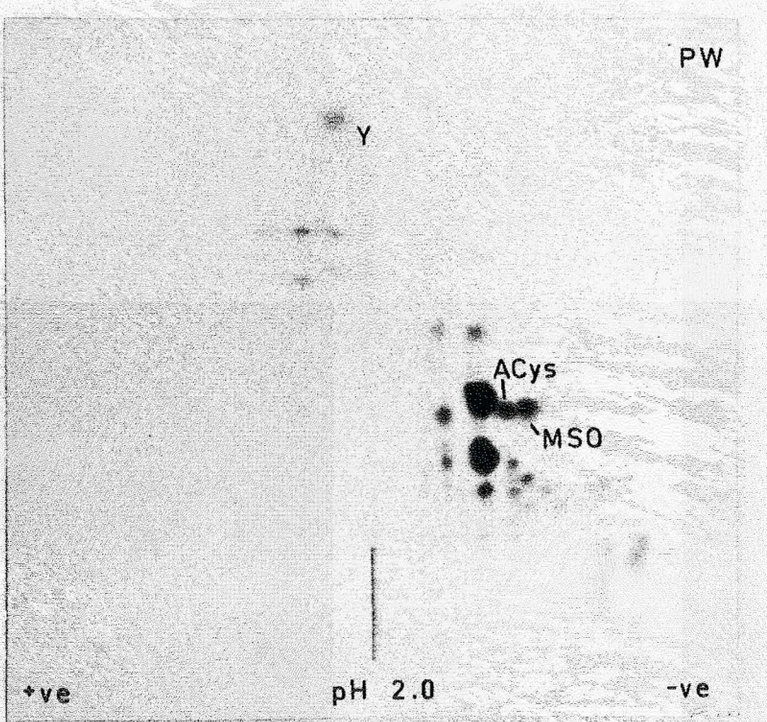
Recovery of radioactivity from the extracts after fractionation

Table 4-22 contains the total counts per minute contained in each fraction and in the extract. Since the % recovery fluctuated above and below 100, errors of measurement were most likely responsible for the recoveries lower than about 100 and so most of the radioactivity was eluted from the columns. This applied particularly to the sulphate present in the HCl fraction of the [(^{35}S) sulphate] treatment because the HCl fraction contained the largest proportion of radioactivity. The recovery of radioactivity from the cation columns could not be estimated to any degree of accuracy since it was only about 10% of the total. Nevertheless the small proportion of the radioactivity in the BF and the relative constancy of the fraction found in the NF suggest rather that the radioactivity in one of the NF's for Experiment 21 was reduced by incomplete transfer from the flasks in which the extracts and fractions were evaporated. This explanation was suggested from rinsing only the lower surface of the round bottom flask in Experiment 21. It was considered the salts would precipitate mostly at the end of evaporation and so the larger portion would be transferred.

FIGURE 4-22 iiii



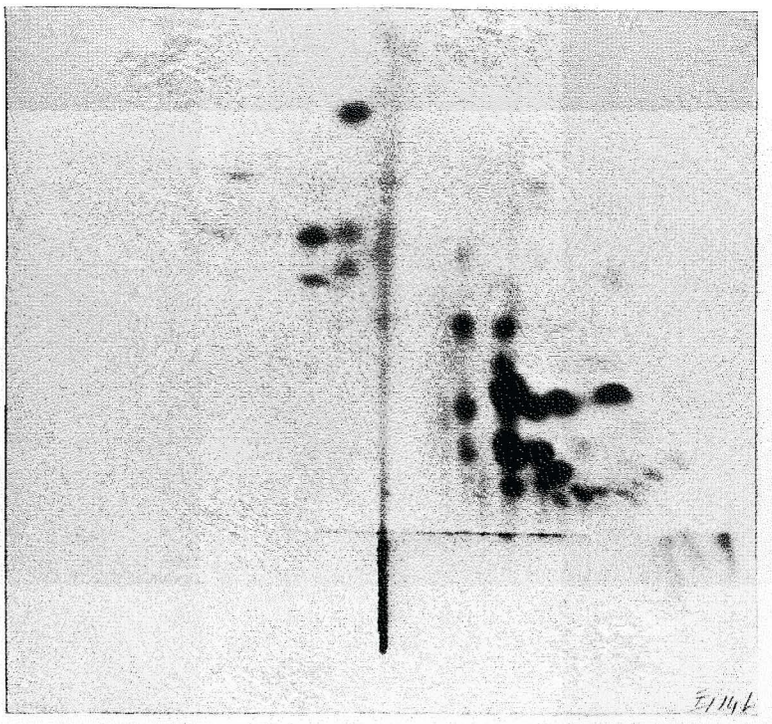
(a) BF OF EXTRACT



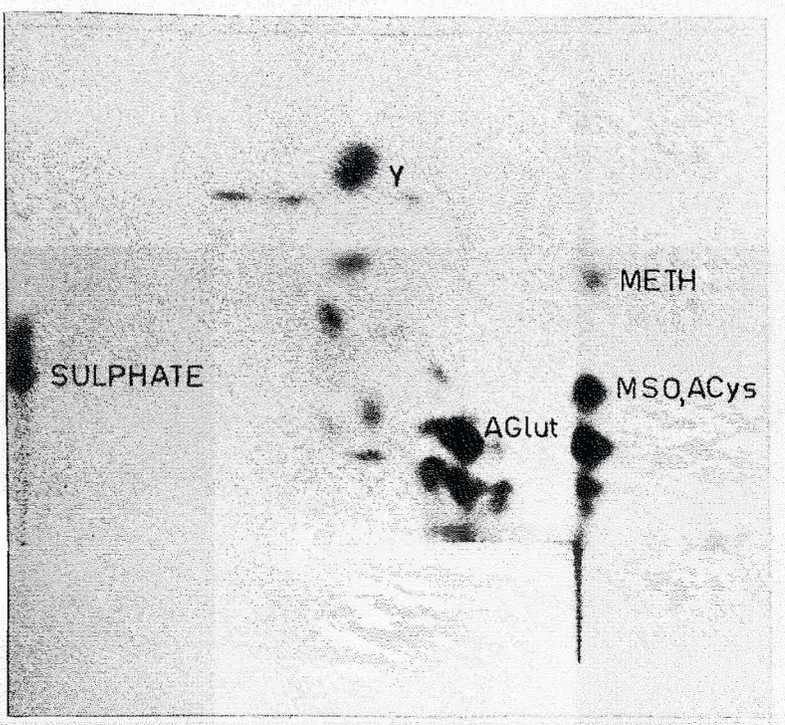
(b) NF OF EXTRACT

FIGURE 4-22iii AUTORADIOGRAMS OF THE BF AND NF FROM AN EXTRACT OF TOMATO ROOTS INCUBATED WITH (^{35}S) SULPHATE FOR ONE HOUR.

FIGURE 4–22 iii



(a) (^{35}S 1/2 hr.) TREATMENT.



(b) ((^{35}S)SULPHATE 1 hr.) TREATMENT. Identification of spots based on their positions only.

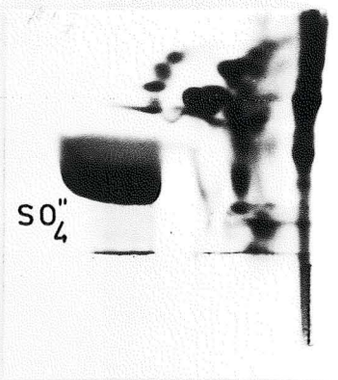
FIGURE 4-22 iii. NF'S OF EXTRACTS FROM TOMATO ROOTS.

FIGURE 4–22 ii

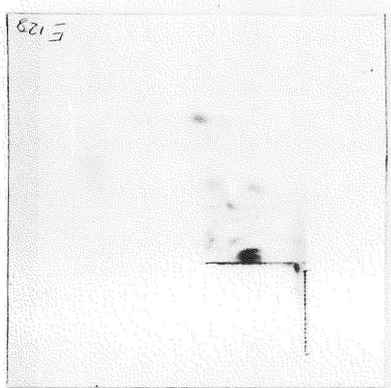
+ve ⟵ pH 5.3 ⟶ -ve

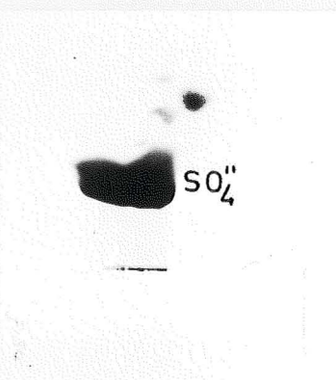
PW ↑

HF. FRACTION (^{35}S AND EXTRACT) HCl FRACTION



HF. FRACTION (^{35}S 1 HR.) HCl FRACTION

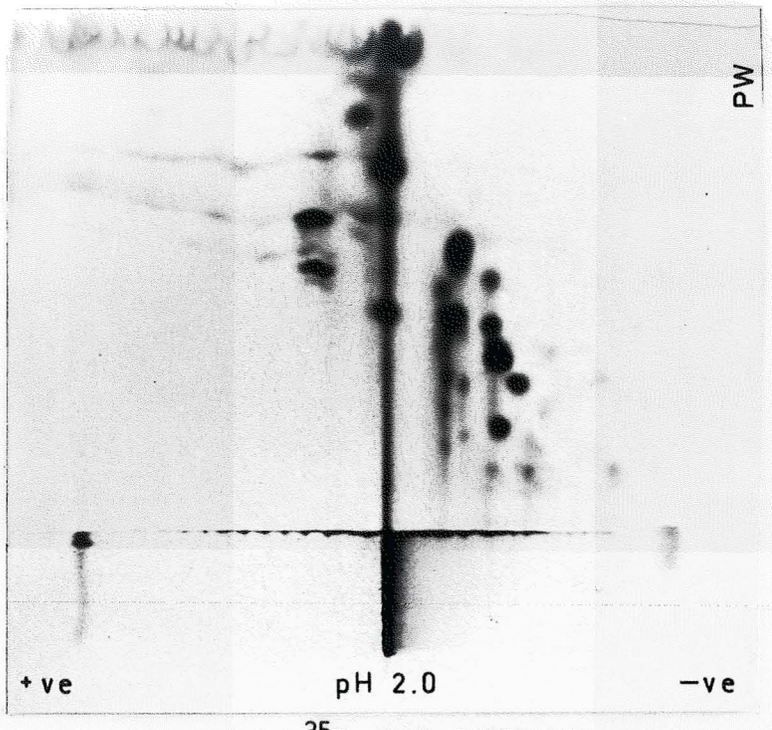




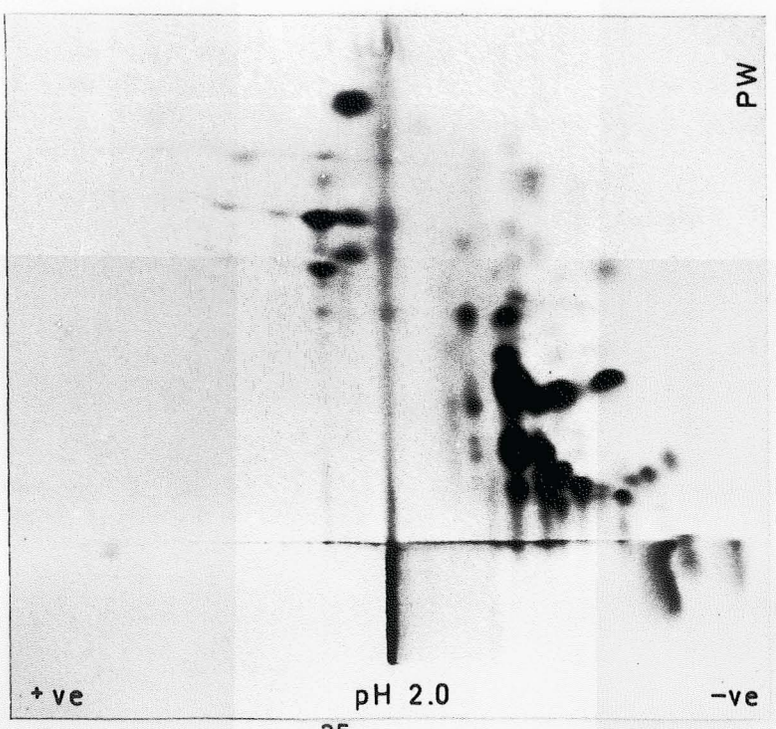
HF. FRACTION ((^{35}S) SULPHATE 1 HR.) HCl FRACTION

EXPERIMENT 22. AUTORADIOGRAMS OF THE HF. AND HCl FRACTIONS FROM TOMATO ROOT EXTRACTS.

FIGURE 4—22 i



(a) (^{35}S AND EXTRACT)



(b) (^{35}S 1 HR.)

EXPERIMENT 22. AUTORADIOGRAMS OF NF'S FROM TOMATO ROOT EXTRACTS.

The labelling of compounds

Figure 4-22i shows autoradiograms of the NF's of the [^{35}S 1 hr.] and [^{35}S and extract] treatments. Figure 4-22iii (a) contains the autoradiogram of the [^{35}S 1/2 hr.] treatment which was similar to the 1 hr. treatment. Since several of the spots in the [^{35}S] treatments appear in the [^{35}S and extract] treatment, it cannot be concluded that those present were formed by the roots.

In Figure 4-22ii are shown autoradiograms of the pH 5.3/PW separations of the HCl fractions from the [^{35}S 1 hr.] and [^{35}S and extract] treatments. Present in both was (^{35}S) sulphate, but the quantity in the [^{35}S 1 hr.] treatment was much greater. This could have been formed either in the roots or in the medium and taken up by the roots. A number of other anionic compounds are also present in the HCl fraction of the [^{35}S 1 hr.] treatment.

Figure 4-22iv shows autoradiograms of pH 2.0/PW separations of the BF and NF from the (^{35}S) sulphate treatment. Although the column was washed with 35 ml. water before eluting the BF, this contained several of the compounds present in the NF and two others - one more basic. Figure 4-22iii (b) shows an autoradiogram of a pH 5.3/PW separation of the NF of the (^{35}S) sulphate treatment. (^{35}S) sulphate was present although the lipid-like material had been removed with MCW. It is possible that this sulphate was derived from the breakdown of a sulphur compound.

Experiment 23

The oxidation of elemental sulphur in 'high magnesium' medium

Since Experiment 22 showed that sulphate was formed during incubation of roots with ^{35}S, a direct determination of oxidation in the medium was attempted.

Experimental details

It was found in Experiment 22 that sulphur anions were present in the water extract of a ^{35}S solution in chloroform. After several washings of the water extract with chloroform the radioactivity did not decrease. It was considered that the oxidation of elemental sulphur could be determined by removing the elemental sulphur from the medium with chloroform and measuring the radioactivity left in the medium. To be sure that the extraction was complete and that the anions were not removed in this procedure, the radioactivity of the same medium was determined after several successive extractions.

To four portions of 25 ml. of sterile 'high magnesium' medium in a 250 ml. beaker was added 64 µg. ^{35}S in 0.8 ml. of acetone. The medium was incubated for 0, 1, 3 and 8 hr. at 26°C.. After the appropriate time, each portion of medium was shaken with 10 ml. 'Pronalys' chloroform in a 250 ml. separatory funnel, the layers were allowed to separate and the chloroform run out. After extracting for at least four times the radioactivity was determined on an aliquot by scintillation counting.

To 0.9 ml. of double distilled water in a vial (obtained especially for scintillation counting) was added a 100 µl. aliquot of medium after extraction. 10 ml. of Bray's solution (1960) was added to the vial and the latter closed. The composition of Bray's solution is shown in Appendix 4. To find the per cent oxidation of the sulphur, 10 µl. of the ^{35}S acetone solution was added to 0.9 ml. of water followed by 10 ml. of Bray's solution. The samples were counted in a Packard 'Tricarb' set for maximum efficiency and the counts corrected for background. The external standard ratios for all samples agreed within 1%

which indicated equal counting efficiency and so all samples were directly compared.

Results and Discussion

The results are shown in the following table:

Time of incubation at 26°C. (hr.)	cpm. in 100 µl. of medium No. of extractions with $CHCl_3$		
	4	5	6
0	195	-	-
1	-	178	-
3	224	-	-
8	157	143	128

800 µl. of the ^{35}S acetone solution contained 1.964×10^6 cpm.

After 8 hr. incubation, the residual radioactivity was least, but each successive extraction after the fourth reduced the radioactivity by about 10%. Now if elemental sulphur were being removed by the extractions, the percentage loss would drop much more quickly since it is insoluble in water and soluble in chloroform. Since sulphate is insoluble in chloroform and very soluble in water the removal of 10% of the radioactivity each time precluded its contributing to the removed radioactivity. Vaseline or silicone grease from the stopcock could have crept over the glass and dissolved some of the elemental sulphur so that the ^{35}S was slowly removed.

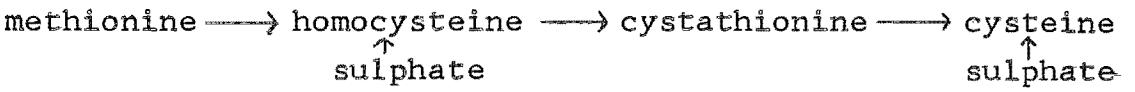
However, regardless of the cause, the results are too variable to draw reliable conclusions. For instance if it is assumed that the rate of oxidation did not exceed 50 cpm in 8 hr. then 2 µg. sulphate-S would have been formed in 168 hr. = 7 days. An average root in Experiment 11 required 4-5 µg. sulphate-S but the difference between the two figures is hardly enough, considering the variability of the cpm, to conclude that oxidation of sulphur in the medium alone was not responsible for the growth of the roots.

Discussion

Excised roots of tomato were found to assimilate sulphate, cystine, homocystine and methionine, and from this it is concluded that cysteine and methionine in protein are synthesized from any of the four compounds. Methionine, cysteine and glutathione were among the compounds formed from (^{35}S) sulphate.

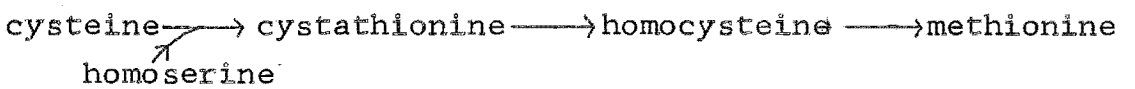
The labelling of soluble methionine but not protein methionine when both (^{35}S) sulphate and methionine are fed is explained by either two methionine pools being present or a greatly expanded methionine pool which therefore contained sufficient radioisotope to be detected, in contrast to the protein hydolyzate, so increasing the detection efficiency. In either case the amount of methionine formed from sulphate was reduced. In methionine synthesis by E.coli, growth on methionine represses the cystathionine forming enzymes: homoserine-O-transsuccinylase and cystathionine synthetase (γ-replacement) as well as the enzyme synthesizing methionine from homocysteine. Methionine also inhibits the activity of homoserine-O-transsuccinylase (Rowbury and Woods, 1961,1966). Although the control of methionine synthesis in higher plants is unknown it could be similar to that in E.coli.

On the other hand the presence of sulphate in the tomato roots prevented the formation of cysteine from methionine. This inhibition was unlikely to be brought about by repression since the change was established well within an hour after the roots had been transfered from methionine to methionine and sulphate. If the enzymes forming cysteine were not inhibited then the small incorporation of radioisotope from methionine into cysteine is inconsistent with homocysteine being the point of entry for sulphide. The amount of labelling of cysteine would have been the same as that of homocysteine which is closely related to methionine, so the quantity of radioactivity in cysteine would probably have been greater had homocysteine been formed before cysteine.



However if the enzymes transforming methionine to cysteine were inhibited by a metabolite of sulphate then the transformation of methionine into homocysteine could have been as easily inhibited as any of the other reactions between the two and so it is not possible to distinguish between cysteine or homocysteine from this evidence.

The formation of methionine from cysteine most likely involved cystathionine and homocysteine.



The growth of the roots in cystine (after it had been reduced to cysteine), from which they must have formed methionine, and their growth on homocystine is consistent with the synthesis of cystathionine and then its degradation to homocysteine as in spinach extracts (Giovanelli and Mudd, 1966 a and b). The role of cystathionine in methionine synthesis and thus cysteine's is also supported by the reduction of glucose incorporation into methionine by homoserine, cystathionine and homocysteine in Paul's Scarlet Rose tissue (Dougall and Fulton, 1966) and by the incorporation of homoserine into methionine in horseradish (Chisholm and Wetter, 1966). Hence the utilization of cystine strongly supports such a pathway in tomato roots.

Although the synthesis of homocysteine as well as, or in place of, cysteine from sulphide is feasible, the role of organic sulphur compounds in the interconversion of cysteine and homocysteine in Neurospora and yeast (Delavier-Klutchko and Flavin, 1965) suggests rather that there is an advantage in the sulphur being bound to a carbon atom during the transformation. Since sulphite is most likely bound during sulphate reduction and sulphide might also be, since external sulphide is oxidized is sulphate before incorporation into cysteine (Wetter, 1964), sulphate reduction

can be envisaged as taking place in a functional unit in which the formation of cysteine is the step at which a free intermediate first appears. The location of a sulphate activating enzyme in chloroplasts (Asahi, 1964) and the presence of sulphite reductase in mitochondria and chloroplasts (Mayer, 1967) of higher plants supports this. In this case the presence of both homocysteine synthetase and cysteine synthetase in the sulphate reduction 'chain' might not be structurally feasible so that these two amino acids are converted through cystathionine as described before.

The assimilation of methionine for the synthesis of cysteine and glutathione and the growth of tomato roots with homocystine is consistent with the fungal and mammalian pathway operating in these roots. Although Giovanelli and Mudd (1966a) suggested that this pathway did not operate in extracts of spinach leaves, the conversion of methionine into cysteine in radish leaves (Thompson and Gering, 1966) is consistent with this transsulphuration. The evidence for tomato roots strongly suggests that sulphate is not involved in the interconversion. The toxicity of methionine to the roots supports a slow rate of conversion into sulphate or, for that matter, into cysteine since such a conversion, especially to sulphate would be a detoxification mechanism. A shunt from homocysteine to cysteine through sulphide is unlikely since the homocystine cleavage enzymes produce free inorganic sulphur (Flavin, 1964; Tishel and Mazelis, 1966) and this would be inconsistent with the postulated bound intermediates of sulphate reduction.

Although several mutants of E.coli will grow with methionine (Lampen, Roepke and Jones, 1947), that this was a sole sulphur source seems unlikely. These authors added sulphate to the medium and Roberts et al. (1955) working with the same mutants used by Lampen et al. found that the (^{35}S) label of (^{35}S) methionine was not transfered to cysteine even under starvation conditions. The bacteria grew a little with methionine as a sole sulphur source and

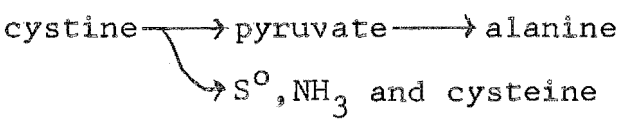
used glutathione and soluble protein sulphur for the synthesis of protein cysteine. In Proteus mirabilis, Grawbow and Smit (1967) found that S-methylcysteine which was present in wild type and mutant cells was an alternative sulphur source for mutants blocked after cysteine synthesis. Thus an alternative pathway for the synthesis of methionine which did not involve cysteine was considered likely by these authors. An alternative pathway to cysteine was also proposed in Neurospora by Ragland and Liverman (1956). In both organisms, the pathway is not involved in the biosynthesis of methionine from sulphate. E.coli was also found by Grabow and Smit to contain S-methylcysteine. If Proteus resembles E.coli and Salmonella in failing to carry out transsulphuration from homocysteine to cysteine (Delavier-Klutchko and Flavin, 1965) then the existence of an alternate pathway of methionine biosynthesis as in Neurospora is not associated with the latter's conversion of methionine into cysteine. Also Thompson and Gering (1966) found that radish leaves formed S-methylcysteine from methionine via cysteine so cysteine biosynthesis from methionine is most unlikely to involve S-methylcysteine.

The most plausible explanation of the utilization of methionine for the synthesis of cysteine by tomato roots is its transformation through homocysteine and cystathionine as in the fungal and mammalian pathway. Although the evidence is not as definitive as it could be, the following is proposed as the pathway of sulphate assimilation in tomato roots.

$$SO_4'' \longrightarrow SO_3'' \longrightarrow S'' \longrightarrow \text{cysteine} \longrightarrow \text{cystathionine} \longrightarrow \text{homocysteine} \underset{\text{methionine}}{\swarrow}$$

The significance of the pathway forming methionine from cysteine is apparent from the latter's synthesis from sulphate but what is the significance of the reverse pathway? Three situations are proposed to explain this.

Firstly, only cysteine and not homocysteine might be degraded to sulphate during hydrolysis of proteins. This is suggested by Tishel and Mazelis (1966) reporting that cystine is degraded much more rapidly than homocystine by a particulate fraction of cabbage extracts. Also Thompson and Gering (1966) found that radish and bean leaves converted (^{14}C) cystine into alanine and ascribed this transformation to the action of cystine lyase.



In pea seedlings, Mothes (1939) found that organic sulphur was converted into sulphate during mobilization of the reserves of the seeds.

Secondly, the presence of a large quantity of glutathione in tomato roots suggests that it might function as a storage compound for sulphur. Fujiwara and Torii (1956) found that the soluble sulphur fraction of barley plants fell almost to zero in the shoots and dropped to about a half in the roots when the plants were sulphur deficient. The per cent sulphate of the soluble fraction also fell. It thus appeared that this soluble fraction was an internal sulphur resevoir. If the soluble sulphur of tomato acts similarly then the glutathione would be a reserve of sulphur along with sulphate, and excess methionine would be converted into cysteine for the synthesis of glutathione. Davies (1966) concluded that the level of methionine regulated respiration in turnip tissue and in this way could its level be controlled.

Thirdly, methionine might be taken up by the plant roots as a sulphur source from soil solution. Formin and Astakova (1959) reported that mustard and rice plants utilized methionine for growth and produced a greater quantity of dry matter than sulphate fed plants. Although the plants were not sterile, they were fed methionine for only 5 hr. each day and the nutrient solution was changed

every day so that microbial transformation probably would have been unimportant. They also found that sterile corn plants took up (^{35}S) methionine and translocated it to the leaves.

Although there have been few reports of soluble sulphur amino acids present in soil, they have been isolated from soil hydrolyzates (Freney, Barrow and Spencer, 1962). Alexander (1961) concluded in a review of organic nitrogen mobilization in soil that proteins were hydrolyzed by microorganisms to amino acids before the latter were oxidized to ammonia. Of the protein amino acids, methionine is the most resistant to this oxidation so that plants may be able to compete with microorganisms for methionine to satisfy some of their sulphur requirements. If this is so then the fungal ability to transform methionine into cysteine would also have nutritional significance.

In this investigation of sulphate assimilation, besides the metabolism by tomato roots of (^{35}S) sulphate and (^{35}S) methionine, several potential sulphur sources were first examined. Although excised roots have the advantages that they are sterile, grow rapidly, require a small inoculum, which contained only a small reserve of sulphur, and are readily measured, the range of sulphur compounds which they assimilated was small and of these only sulphate was without complications. Methionine in spite of giving good growth at lower concentrations appeared to alter the fresh weight yield related to the supply of sulphur and was toxic at relatively low concentrations, slightly above those utilized for growth. Cystine and homocystine were poorly assimilated compared with sulphate. Although glutathione was present in the roots in considerable quantity they appeared to be unable to incorporate the cysteine of exogenous glutathione into protein.

The roots also required a relatively large volume of medium and a considerable quantity of sugar, both of which made reduction of the sulphur impurity difficult. If the investigation is to be carried forward then the culture of

Paul's Scarlet rose tissue in suspension culture (as reported on by Dougall, 1966, 1965) would have advantages over excised roots. The shake cultures are composed of similar types of cells in contrast to roots, and require similar supplements to tomato roots (except for a requirement for glutamine) as well as less medium. Although the fresh weight gain was about half that for tomato roots, this slight disadvantage is overruled by the incorporation of a wide range of amino acids for protein synthesis in contrast to tomato roots (Street, 1966); so that Isotope competition experiments could then be used, as well as more extensive feeding experiments with radioisotopes.

The present evidence is consistent with cysteine and not homocysteine being the primary sulphur amino acid and more conclusive proof requires that homocysteine be identified and that at least the radioactivity to be found in it be reduced more than that in cysteine by methionine feeding. The failure to detect acetamido-homocysteine prevented such a comparison. The apparent absence of acetamido-homocysteine is possibly brought about by a greater instability than that of acetamidocysteine. This unexpected decomposition of acetamidocysteine alone is a serious drawback to the use of iodoacetamide for the stabilization of thiols since it renders a fluctuating recovery of radioactivity possible in several compounds. N-ethylmaleimide has been more extensively used for the protection of thiol groups (Ellis, 1963, 1966) so that its use in future work is indicated. If a similar ion exchange fractionation to that employed here were to be used, then the neutral amino acid fraction would have to be eluted with hydrochloric acid instead of ammonia to avoid cyclysation of the NEM cysteine derivative. NEM derivatives of thiols have the advantage that they have greater Rf's than acetamido derivatives in Propanol-water, so removing them from the other sulphur compounds present. This would greatly improve the separation between glutathione and cysteine present as acetamido-derivatives.

Perhaps the most pressing problem is the assessment of the stability of several of the sulphur compounds labelled, more especially those related to methionine biosynthesis. The continual presence of (^{35}S) sulphate in the neutral amino acid fraction suggests that some of these compounds broke down. Elution and separation again would indicate those derived from others. Once reproducible recovery can be established then measurement of the radioactivity in the compounds will present the clearest evidence for the pattern of sulphate assimilation.

In further investigations of these compounds, as short a period as possible between feeding the radioisotope and the separation of the compounds would reduce the formation of artifacts. The ion exchange of extracts, although allowing a separation, increases the time between harvesting and examination of the extracts, but the choice of another tissue which does not contain substances interfering in electrophoresis and chromatography would circumvent this. Since it was established that an impurity in the layer was responsible for much of the streaking in a purified extract, a direct separation of an extract on a thin layer by electrophoresis and chromatography may be possible.

The labelling of sulphur amino acids was considerable after fifteen min. with carrier free (^{35}S) sulphate, so the establishment of the early formed compounds in sulphate assimilation should be feasible. This along with the affect of cystine and methionine on the flow of the label from (^{35}S)sulphate should provide evidence of the roles of cysteine, homocysteine and glutathione, which can be further examined by enzymic studies.

Once the main pathways of metabolism are established it will be possible to examine more critically the nutrition of the intact plant and to predict more accurately a need for fertilizer as well as to increase the synthesis of protein cysteine and methionine for the nutrition of animals. Although the interrelationships among sulphate, cysteine and methionine are complex, their elucidation should be rewarding.

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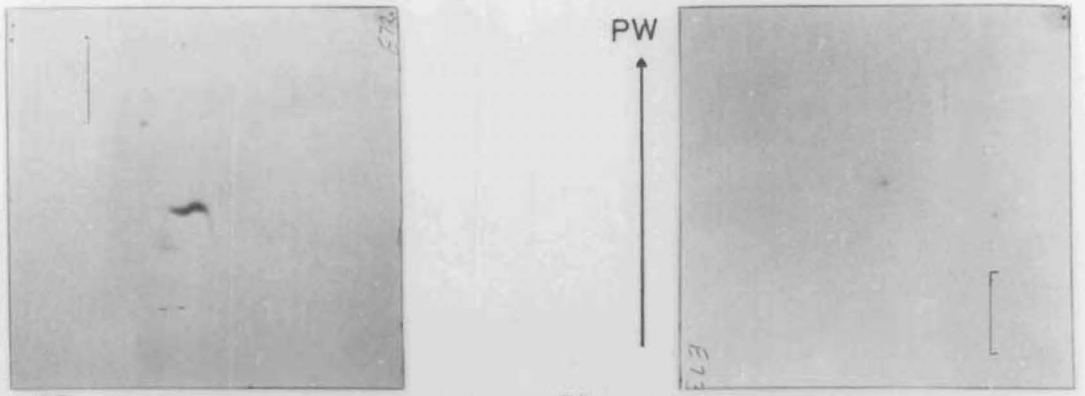
APPENDIX FIGURE 1



([^{35}S]SULPHATE AND EXTRACT) ([^{35}S]METHIONINE AND EXTRACT)

EXPERIMENT 20

([^{35}S]METHIONINE 1 HR.) ([^{35}S]SULPHATE 1 HR.)

EXPERIMENT 21

([^{35}S]SULPHATE 1/4 HR.) ([^{35}S]SULPHATE 1/2 HR.)

EXPERIMENT 21

EXPERIMENTS 20 AND 21. AUTORADIOGRAMS OF HF FRACTIONS FROM TOMATO ROOT EXTRACTS.

Appendix 1-1

The preparation of Media

Media for clonal maintenance or for culture experiments were prepared immediately before use from the required stock solutions of the vitamins, tryptophane, the inorganic nutrients and from freshly prepared solutions of sugars and yeast extract. All salts were 'Analar', except for LR calcium nitrate, EDTA, and ferric tartrate (B.P.), unless otherwise stated.

The concentrations of the stock solutions shown here as X10S, X100S or X1000S refer to the concentrations of these stock solutions relative to the final concentration of the medium, so that for a solution at X100S, 10 ml. of this solution was required for each litre of final medium.

The preparation of stock solutions

Vitamin supplements

The stock solution of vitamin supplements contained:

1.2 g. glycine, B.P.
0.04g. thiamine HCl, B.P.
0.04g. pyridoxine, B.P.
0.2 g. nicotinic acid, B.P.

in 400 ml. of solution prepared with double distilled water. This was stored at -15°C. in 5 and 10 ml. aliquots in plugged test tubes. When required, 1 ml. of thawed vitamin stock solution was used per litre of final medium.

Charles and Street's micronutrients used with Bonner's (1940) medium in media III, IV, V, VI, VII, VIII.

Two variants of Charles and Street's micronutrients were used: the first was used until it was found that a sufficient quantity of sulphur was present in minus sodium sulphate media to allow maximum growth of Petkus rye roots. The second was equivalent except that the sulphate salts were replaced by their respective chlorides.

Appendix 1-1

First Variant:- A

$ZnSO_4.7H_2O$	0.265 g.
KI	0.075 g.
H_3BO_3	0.150 g.
$MnCl_2.4H_2O$	10 mg.
$CuSO_4.5H_2O$	2 mg.
H_2MoO_4	50 ml. of a solution of H_2MoO_4-6.8 mg. in 2000 ml. DDW

The solution was made up to 1000 ml.

Second Variant:- B

This was the same as A except that the sulphate salts were replaced with the following salts:

ZnO 75.2 mg. dissolved in a minimum quantity of 1N HCl or

20 ml. of a solution of 752 mg. ZnO, dissolved in 1N HCl, and made up to 200 ml. of solution with double distilled water.

$CuCl_2$ 1.36 mg. B.P.

The solution was made up to 1000 ml.

For each variant the pH was raised to around 4.5 before making the solution up to volume. 10 ml. of stock solution was used for each litre of medium.

Appendix 1-2

Media for the growth of rye roots

Medium I - Charles and Street's (1959)

The macro- and micronutrient components given below plus 2% glucose, unless otherwise stated, plus vitamin supplement as given before and plus yeast extract.

The stock solutions were prepared as follows:
Each of the salts was dissolved in double distilled water.

Sol. A	$Ca(NO_3)_2.4H_2O$	144 g.
	KNO_3	40 g.

The solution was made up to 500 ml.

Sol. B	KCl	32.5 g.
	NaH_2PO_4	10.75g.

The solution was made up to 500 ml.

Sol. C	$MnCl_2 4H_2O$	78.5 mg.
	$ZnSO_4.7H_2O$	1.32g.
	KI	0.375g.

The solution was made up to 500 ml.

Sol. D	$Na_2SO_4.10H_2O$	90.7 g.
	$MgSO_4.7H_2O$	148.0 g.
	H_3BO_3	0.30g.

The solution was made up to 2000 ml.

Sol. E	H_2MoO_4	6.8 mg. in 2000 ml. of water.
Sol. F	$CuSO_4.5H_2O$	16 mg. in 2000 ml. of water.

Stock solution of Iron

0.5 ml. of 60% w/v $FeCl_3$ solution, 'analar'.

0.5 g. Na_2EDTA

make up to 1000 ml of solution. Use 10 ml. for a litre of medium.

A solution of X10 inorganic stock for immediate use was prepared from these as follows.

50 ml. of solutions A, B and C; 500 ml. of solution D; 25 ml. of solution E; and 125 ml. of solution F; were added successively to 1.5-2l. of double distilled water in a 5l. flask, and the solution was well mixed after each addition. The X10S solution was made up to 2l. in a measuring cylinder and then 3l. of double distilled water was added. This stock was stored at less than $5^{o}C$. It was diluted 10 times when medium was prepared, and the Iron Stock added at that time.

Medium II - Murashige and Skoog's (1962) medium

The macro- and micro-nutrients given below plus 2% glucose, vitamin supplement and plus 30 ppm yeast extract. Charles and Street's Iron stock was used in place of Murashige and Skoog's.

NH_4NO_3	16.5 g. in 100 ml. of solution
KNO_3	19.0 g. in 100 ml. of solution
$MgSO_4.7H_2O$	3.7 g. in 100 ml. of solution
KH_2PO_4	1.7 g. in 100 ml. of solution
$CaCl_2.6H_2O$	6.56g. in 100 ml. of solution

Use 10 ml. of each of the above solutions for a litre of medium.

KI	80 mg. in 100 ml. of solution
$MnSO_4.4H_2O$	2.23 g. in 100 ml. of solution
$ZnSO_4.7H_2O$	1.06 g. in 100 ml. of solution
H_3BO_3	0.62 g. in 100 ml. of solution
$CuSO_4.5H_2O$	2.5 mg. in 100 ml. of solution
H_2MoO_4	1.6 mg. in 100 ml. of solution
$CoCl_2.6H_2O$	0.25 mg. in 100 ml. of solution

Use 1 ml. of the above solutions for each litre of medium.

Each of these stocks was added individually to the medium. If 10 ml. or more of a stock solution was required, it was dispensed from a 25 or 50 ml. measuring cylinder. If less than 10 ml. was required the solution was pipetted from a beaker.

Medium III - 'rye clone' medium

The inorganic components of Bonner's (1940) medium plus Charles and Street's micronutrients A or B prepared in one stock solution as shown before. The medium also contained 2% glucose, vitamin supplement and yeast extract at 60 ppm, unless otherwise stated.

Stock Solutions

Each of the salts were dissolved in double distilled water.

KCl	6.5 g.
KNO_3	8.1 g.
$Ca(NO_3)_2.4H_2O$	23.6 g.

The solution was made up to 1000 ml.

$MgSO_4.7H_2O$	3.6 g.

The solution was made up to 1000 ml.

KH_2PO_4	2.0 g.

The solution was made up to 1000 ml.

Ferric tartrate B.P. 0.3 g.

The solution was made up to 2000 ml.

These stocks were stored separately at less than 5°C. To prepare the medium 10 ml. of each of them was used for a litre of medium.

Medium IV - 'tryptophane' medium

The inorganic components of Bonner's (1940) medium with the calcium and potassium nitrates replaced with sodium nitrate at a lower concentration of nitrate and with their respective chlorides. Magnesium sulphate was replaced with magnesium chloride and sodium sulphate and Bonner's ferric tartrate was replaced with iron EDTA. The medium also contained Charles and Street's micronutrients B, 0.54 ppm tryptophane, 2% glucose, and vitamin supplement.

Stock Solutions

KCl	12.4 g.
$NaNO_3$	15.3 g.
$CaCl_2.6H_2O$	21.9 g.
$MgCl_2.6H_2O$	2.96g.

This solution was made up to 1000 ml.

KH_2PO_4 2.0 g.

This solution was made up to 1000 ml.

Na_2SO_4 2.07g.

This solution was made up to 1000 ml.

FeEDTA: 0.175 ml. of $FeCl_3$ 60% sol.

0.115 g. Na_2EDTA

The solution was made up to 1000 ml.

A sample of the anhydrous sodium sulphate was dried in an oven at 160°C and found to lose less than 1% in weight.

These stocks were stored separately at less than 5°C. To prepare the medium, 10 ml. of each of them was used for a litre of final medium.

Appendix 1-3

Media for the growth of tomato roots

Medium V - 'tomato clone' medium

The inorganic components of Bonner's (1940) medium as for medium III, and as set out below. The medium also contained Charles and Street's micronutrients B, 1.5% sucrose and Vitamin supplement.

Stock solutions

Each of the salts were dissolved in double distilled water.

KCl 6.5 g.

KNO_3 8.1 g.

$Ca(NO_3)_2.4H_2O$ 23.6 g.

The solution was made up to 1000 ml.

$MgSO_4.7H_2O$ 3.6 g.

The solution was made up to 1000 ml.

KH_2PO_4 2.0 g.

The solution was made up to 1000 ml.

Ferric tartrate 0.3 g.

The solution was made up to 2000 ml.

These stocks were stored separately at less than 5°C. To prepare the medium 10 ml. of each of them was used for a litre of final medium.

Medium VI - 'low N' medium

The same modified Bonner's stocks of medium IV were used for this medium. The medium also contained Charles and Street's micronutrients B, 1.5% sucrose and vitamin supplement.

Medium VII - 'high magnesium clone' medium

The inorganic components of Bonner's (1940) medium with added magnesium chloride, as set out below. The medium also contained Charles and Street's micronutrients B, 1.5% sucrose and vitamin supplement.

Stock solutions

Each of the salts were dissolved in double distilled water.

KCl	6.5 g.
KNO_3	8.1 g.
$Ca(NO_3)_2.4H_2O$	23.6 g.

The solution was made up to 1000 ml.

$MgSO_4.7H_2O$	3.6 g.
$MgCl_2.6H_2O$	56.3 g.

The solution was made up to 1000 ml.

KH_2PO_4	2.0 g.

The solution was made up to 1000 ml.

Ferric tartrate	0.3 g.

The solution was made up to 2000 ml.

These stocks were stored separately at less than 5°C. To prepare the medium 10 ml. of each of them was used for a litre of final medium.

Medium VIII - 'high magnesium' medium

The modified inorganic components of Bonner's (1940) medium with increased magnesium chloride, and the sodium sulphate containing stock ommitted. The medium also contained Charles and Street's micronutrients B, 1.5% sucrose and vitamin supplement.

Stock solutions

The salts were dissolved in double distilled water.

KCl	12.4 g.
$NaNO_3$	15.3 g.
$CaCl_2.6H_2O$	21.9 g.
$MgCl_2.6H_2O$	59.2 g.

The solution was made up to 1000 ml.

KH_2PO_4	2.0 g.

The solution was made up to 1000 ml.

FeEDTA	0.175 ml. of $FeCl_3$ 60% solution
	0.115 g. Na_2EDTA

The solution was made up to 1000 ml.

Ten millilitres of each of the stock solutions was used for each litre of medium.

Sodium sulphate containing stocks for use with Medium VIII

For the medium containing 117μ g.S/25 ml. the following stock was used:- the same as that for medium IV.

Na_2SO_4	2.07 g.

The solution was made up to 1000 ml.

When the medium was prepared, 10 ml. of the stock solution was added to make a litre of final medium.

For the media containing up to 56μ g.S/25 ml., the following stock solution of sulphate was used.

17.2 ml. of the above sulphate stock was taken and made up to 400 ml. with double distilled water.

This solution contained 20μ g. sulphate-S/10 ml. The appropriate quantity was added to the medium when it was prepared.

Appendix 1-4

Preparation of media

Constituents of the medium were added in the following order to increase the chelation of iron. Either a weighed quantity of sugar or the ion exchanged sugar solution was added to one third of the final volume of double distilled water. The sugar was dissolved before adding the vitamin stock. Yeast extract, when required, was weighed and added; tryptophane was pipetted in from a X100 stock solution. The appropriate volume of iron stock solution was then added and this was followed by appropriate amounts of stock solutions of the remaining inorganic salts. The pH of the medium was adjusted to around 4.8 $\pm$ 0.1 before autoclaving except for those media of which the pH was aseptically adjusted and for Murashige and Skoog's (II) which was adjusted to 5.7. The medium was then made up to final volume if no filter sterilized solution was to be added after autoclaving. In the latter case it was made up to an appropriate volume so that the correct concentration would result on adding the sterile solution. For this purpose any evaporative loss during autoclaving was ignored. This loss was about 1-2 % of the total volume.

Dispensing the medium

After the medium had been made up to final volume, it was dispensed from a buretted graduated in 50, 25 or 20 ml. portions into 100 or 250 ml. flasks. For a series of media in which the concentration of one substance is varied the lowest concentration was dispensed into flasks first and then the next highest and so on for all treatments without rinsing the burette between treatments. A smaller change of concentration results from this procedure than if the burette were rinsed with distilled water between treatments.

Sterile media were dispensed in the transfer room.

Appendix 1-5

Dissolving of sulphur compounds for filter sterilization

Sufficient solution was prepared freshly each time so that the accuracy of weighing was ± 5%. The concentration of sulphur atom was calculated to be 21 µg. in each ml. of solution. Homocystine and cystine, whose solutions were almost saturated at room temperature were dissolved by shaking the solution in an incubator at 30°C. overnight. After solution, the pH was checked and adjusted if necessary to that found for the sulphate control of the experiment. In general it was found necessary to adjust only those compounds containing hydrochloric acid such as cysteine. After addition of the sterilized solution to the bulk medium it was found that the pH agreed with that of the sulphate controls within 0.1 of a pH unit.

Preparation of a colloidal solution of elemental sulphur

The appropriate quantity of a stock solution of elemental sulphur in 'Analar' acetone, stored at less than 5°C. and containing 1 µg.S/12.5 µl. of acetone, was added to 5 ml. of double distilled water in each of the 100 ml. culture flasks to give the quantity of sulphur contained in 25 ml. of final medium. Between 25 and 800 µl. of acetone were added to each flask. The flasks were capped and then autoclaved at 5 lbs. for 5 mins. to avoid melting and coagulating the sulphur. To each flask was added in the sterile room, 20 ml. of the medium which contained the remainder of the constituents at X1.25S and which had been autoclaved in bulk.

Appendix 2

The calculation of the mean, its standard error and 't' for the significance of a difference between two means based on their standard deviations

All the measurements of fresh weight gain for one treatment were summed to give ΣX and divided by the number of roots harvested to give the mean value. Each of the values was squared and the squares summed to give $\Sigma(X^2)$. From ΣX and $\Sigma(X^2)$ the standard error of the mean (SEM) was calculated as shown in the equation, which was derived from Chambers (1958).

$$SEM = \sqrt{\frac{\Sigma(x^2) - \frac{(\Sigma x)^2}{N}}{N(N-1)}}$$

Where N= the number of roots harvested.

The value 't' was calculated from the equation for samples smaller than fifty. (Chambers, p.42 Equation 14A, 1958) shown below.

$$t = \frac{\left|\bar{x}_1 - \bar{x}_2\right| \sqrt{\frac{1}{\bar{N}'}}}{\sqrt{\left\{\Sigma(x_1^2) - \frac{\Sigma(x_1)^2}{N_1} + \Sigma(x_2^2) - \frac{\Sigma(x_2)^2}{N_2}\right\}}}$$

Where $\bar{x}$ is the mean, N is the number of roots harvested, N' is $\frac{N_1 + N_2}{N_1 . N_2 (N_1 + N_2 - 2)}$, and the subscripts 1 and 2 refer to the two treatments being compared. The number of degrees of freedom is $N_1 + N_2 - 2$.

Appendix 3-1

Preparation of Thin layer plates

Silica gel H (Merck), without binder, Cellulose powder (Macherey Nagel MN300) and distilled water, in the proportions described by Bieleski and Turner (1966) (4:10:80 by weight), were homogenized in a 'Virtis 45' at full speed (approximately 20,000 rpm) for three minutes, allowed to settle one minute and homogenized again at full speed for a further one minute.

Dry, clean 11.5 x 11.5 cm. 24 oz. glass plates were placed edge to edge on a plastic sheet laid on a level bench. After checking that the edges nearest to the operator were all level, a little distilled water was run under each to prevent movement.

For a spreader, a box was constructed from Perspex 12 cm. wide and 10 cm. long. Pieces of Perspex were glued on to the sides, projecting below the bottom, to guide the spreader along the plates. At the front a sloping piece of Perspex butted against the wall so that the spreader would lift over any irregularities between the plates. The gap was set at 300 microns with screws projecting through the back wall so that the spreader was slightly tilted.

Clean, dry 20 x 20 cm. 24 oz. plates were spread, as described in the manual, on a Shandon Unoplan spreader. The gate was set at 300 microns.

Before the spreader was filled with slurry, it was run along the plates to test for any check to movement and then placed on an end plate. After the slurry had been mixed as above, it was poured into the spreader. This was moved slowly and as smoothly as possible along the plates, without downwards pressure, and off the end of the last plate.

The plates were allowed to air dry and then were cleaned along the edges and bottom from excess slurry. The layer was scraped about 1mm. back from the edges to which it

reached with a razor blade. The plates were then thoroughly air dried in the draft from a fan.

No heat was used to dry the plates after it was found that placing them in an oven at 50 to 60°C. greatly increased the brown colour of the front after chromatography in PW. Before this the plates had been air dried until the layer was white and then finished off at 35°C. in an incubator.

Appendix 3-2

Ascending paper chromatography

Two dimensional

The extract was spotted on to the origin, 2.5 cm. from each edge of a corner, of a 22 x 18 cm. sheet of Whatman No. 1 chromatography paper. The paper was rolled into a cylinder, with the long edges together, and tied with two loops of white cotton. The chromatogram was developed at room temperature (approximately 20°C.) in a bell jar without equilibration, by standing it in a Pyrex Petri dish filled to a depth of about five millimetres with solvent.

After the solvent had reached the top of the paper, the chromatogram was hung to dry from stainless steel film clips in a clean fume cupboard and the air from a fan directed up through it. When dry the paper was rolled up in the other direction, tied with cotton and developed as before.

One dimensional

The procedure for two dimensions was also adopted for one dimension. Chromatograms were 22 cm. wide and 25 cm. long as this was the tallest size that could go under a bell jar. The origin was placed 2.5 cm. from the bottom of the chromatogram.

Descending one dimensional paper chromatography

Chromatography with 51 x 23 cm. sheets of Whatman No. 1 chromatography paper was carried out in Shandon glass tanks at room temperature (approximately 20°C.). The compounds to be separated were spotted 7.7 cm. from a short edge to within 2.5 cm. from the long edges. The chromatograms were run without prior equilibration.

Application of sample to Chromatograms

Solutions were spotted on to the paper chromatograms with Carlsberg pipettes, Pyrex glass cappilaries, and five and ten microlitre microcaps (Drummond Scientific Co.). Thin layer plates were spotted with two microlitre microcaps and glass capillaries. The spots on paper chromatograms were dried with compressed air from a slit in a piece of rigid plastic tubing under the origin. Thin layer plates were dried either with compressed air or a hair drier blowing air at room temperature.

Appendix 3-3

Purification of (^{35}S) methionine

Descending chromatography of methionine was carried out on 51 x 23 cm. sheets of Whatman No. 1 as described in Appendix 3-2. Each sheet was first washed with 100 ml. of 0.2% Na_2EDTA in a chromatography tank. The EDTA was washed out with 400-500 ml. of double distilled water and the paper was air dried.

(^{35}S) methionine was spotted on as a band and 2 spots of marker methionine were placed on each side of this, separate from the (^{35}S) methionine. After the solvent containing 0.2% thiodiglycol had dried, strips were cut off each side of the paper and dipped in ninhydrin solution. A strip of the central piece of chromatogram whose radioactivity was intense and corresponded to the methionine marker, was cut out. The methionine was eluted from this, either into a test tube if further chromatography was

intended or into a cation resin column. Elution of the paper strip was carried out as described by Moses (in Smith, p. 507, 1960) with boiled distilled water containing 0.2% thiodiglycol, at less than 5°C. to reduce oxidation of methionine.

The cation resin column was prepared from Zeocarb 225, 25-50 mesh. The resin in the sodium form was ground with a pestle and mortar until 1 g. was washed through a 70 mesh sieve. Amberlite IRC 50 was also dry sieved through the 70 mesh sieve. The fines were decanted from a settled slurry of the resin. Each was purified of iron and resin breakdown products by digestion with 2N HCl at 100°C for half an hour and then rinsed with distilled water, all as described by Thompson et al. (1959). The resin was then regenerated with excess 1N sodium hydroxide.

The Zeocarb column was prepared from a 'Transpet' plugged in the constricted part with glass wool. 17 mg. of blotted resin (= 20 µl.) was poured into the column and the top of the resin covered with a piece of Whatman No. 1 chromatography paper. The resin was regenerated with 200 µl. of 10% HCl and washed with 2 ml. of boiled distilled water.

The IRC 50 resin was packed in a 5 mm. bore tube joined to a 1 mm. bore capillary tubing which was bent into a siphon to prevent the column running dry. The top of the capillary was plugged with glass wool and 170 mg. of blotted resin added. This quantity is 10 times that theoretically needed as the rate of exchange of H^+ ions in a weak acid resin is about 5 times slower than a strong acid resin (Hopkins and Williams handbook). The top of the resin was covered with two pieces of chromatography paper and regenerated with 2 ml. of 10% HCl then washed with 5 ml. of boiled distilled water. The regeneration and washing of both resins was carried out at less than 5°C.

The eluate from the chromatography paper strip was passed through the Zeocarb resin at less than 5°C and the

latter washed with 200 µl. of boiled distilled water. The methionine was eluted from the resin with two 20 µl. portions of 2 N ammonia and washed with 150 µ l. boiled distilled water into the IRC 50 resin to remove the ammonia. The volume of wash water had been determined by testing drops of the effluent with a monitor until the quantity of radioactivity was low. Then the solution was washed through the IRC 50 resin with 700 µ l. of boiled distilled water. The volume required for this had been found in the same way as for the water washing the Zeocarb resin. The total of 0.9 ml. of (^{35}S) methionine solution was collected in a tapered graduated centrifuge tube.

Appendix 3-4

Oxidation of sulphur compounds

Sulphur compounds were oxidized as described by Peterson and Butler (1962).

Five milligrams of methionine and S- methyl cysteine, and two milligrams of cystathionine were oxidized to the sulphoxides with five millilitres of three per cent w/v of hydrogen peroxide. Five milligrams of methionine and S-methyl cysteine were oxidized to the sulphones and five milligrams of cystine, homocystine and glutathione were oxidized to the sulphonic acids, each with five millilitres of three per cent hydrogen peroxide and 0.02% ammonium molybdate. After the solutions had stood at room temperature for about 15 minuts, they were frozen in a dry ice -ethanol mixture and freeze dried. When the amino acids were dry, each was dissolved in 5 ml. of ten per cent isopropanol, except for cystathionine sulphoxide to which 2 ml. of ten per cent isopropanol was added. The solutions were stored at less than 5°C. in eight-dram vials.

Preparation of Acetamido-derivatives (modified from Ellis, 1966)

In a vial, seven milligrams of cysteine HCl (equivalent to five milligram cysteine) were weighed out and dissolved in 1 ml. of distilled water. The pH of the solution was adjusted to about 6.5 with 0.1N sodium hydroxide. Ten milligrams of glutathione were dissolved in 1 ml. of distilled water. To each of these solutions was added 1 ml. of 0.1M sodium phosphate buffer, pH 7.4, followed by 15 mg. of iodoacetamide dissolved in 1 ml. of ten per cent isopropanol. The solutions were then made up to five millilitres.

To purify Acys, 75 µl of the solution was spotted as a band on a 10 x 8" chromatogram, which had been washed with EDTA, and run in PW by ascending chromatography. After drying the paper, two strips were cut, one from each side, and dipped in ninhdrin solution. A strip corresponding to the ACys ninhydrin spots, was then cut from across the central piece of the chromatogram. This was eluted into a vial with boiled distilled water, as described by Moses (in Smith, p. 525, 1960), at less than five °C until one to two millilitres of solution had accumulated. This was freeze dried and 50 µl. of ten per cent isopropanol added to the residue.

AG1 was purified by cromatography in BPW in the manner described for Acys.

When the purity of these compounds was checked by electrophoresis at pH 2.0, a number of weak ninhydrin spots, positively charged, were detected, all with a greater mobility than ACys and AGlut. The number and mobility of these extra spots was the same for the ACys and AG1 preparations, and they were not affected by peroxide oxidation. These extra spots, which did not appear in electrophoresis runs of the unpurified Acys preparation were thought to be derived from the EDTA, in which the chromatography paper used for purification was rinsed.

Homocysteine was prepared from five milligrams of homocystine by reduction in an electrolytic desalter, similar to the one described by Smith (p. 41, 1960) but without water cooling or flowing mercury electrode. A solution of five milligrams of homocystine in HCl was made up to one millilitre with distilled water and added to the apparatus. After reduction for 20 mins. at 12 mA, the solution was sucked into a vial containing one millilitre of 0.1M phosphate buffer and 15 mg. of iodoacetamide. After several minutes three millilitres of ten per cent isopropanol were added.

The AHys was not separated from homocystine and homocysteine thiolactone, and the solution was stored at less than $5^{o}C$.

Appendix 4

Determination of Radioactivity

The operating voltage of the Geiger-Müller tube was determined as described by Francis, Mulligan and Wormall (1959) p. 387, from a plot of the cpm against the voltage applied to the G.M. tube. A voltage near the middle of the plateau (540) was used for all measurements made in this work. The dead or resolving time of the tube was determined by the method of paired sources as described by Francis et al. p. 226.

At least 1000 counts were accumulated for each sample. Those samples with cpm greater than 10,000 were corrected for the resolving time of the instrument from the nomogram in Francis et al. p. 225. All observed counts were corrected for the background (about 15 cpm) determined on the same day and the cpm were corrected for the decay of the radioisotope from the day on which the experiment was carried out, except for Experiment 23.

The efficiency of counting was determined with a portion of diluted carrier free (^{35}S) sulphate supplied by Amersham. Three aliquots were pipetted onto aluminium planchets and their radioactivity found to agree within 3%. The average was used to calculate the efficiency. Three aliquots were also counted on stainless steel planchets and these were found to have a higher counting efficiency, presumably brought about by increased backscattering. It was assumed that at all rates of decay the same fraction of electrons would be backscattered. From the observed count on stainless steel was calculated a factor to convert the HCl fractions counted on stainless steel planchets into the rate for aluminium.

Bray's solution (1960) contained the following constituents:

naphthalene	6.0 g. (L.R.)
PPO	0.4 g.
POPOP	0.02g.
methanol	10.0 ml. ('Analar')
ethylene glycol	2.0 ml. (L.R.)

p-dioxane: add to the above solution to make 100 ml.

The solution was stored at less than $5^{o}C$..

Appendix Table 5 - 1

The growth of rye roots in Street and Charles' medium (I) with varying concentrations of yeast extract.

Concentration of YE/ppm	Passage Number	Increase of main axis (mm.)	Lateral number	Lateral length (mm.)	No. harvested.
40	1	44.7 ±2.9	20.9±2.0	179±24	10
	2	42.3 ±1.8	23.8±0.8	319±21	10
60	1	41.8 ±1.9	24.6±1.3	309±34	10
	2	41.4 ±1.3	23.8±0.8	319±21	10
80	1	33.6 ±1.7	16.0±0.9	202±20	10
	2	32.0 ±1.7	16.7±1.4	241±27	10
100	1	33.3 ±1.4	19.3±1.2	247±14	20
	2	31.3 ±1.1	17.2±0.6	257±10	20
120	1	34.2 ±2.3	17.4±1.7	251±12	9
	2	30.0 ±1.7	15.5±2.0	251±28	8
140	1	32.3 ±2.0	15.5±1.4	228±11	10
	2	28.9 ±2.2	13.3±1.1	224±19	10
160	1	27.6 ±2.2	11.0±2.0	156±27	10
	2	23.2 ±1.7	10.1±1.3	161±14	9

Appendix Table 5 - 2

The growth of rye roots with several sulphate concentrations.

'Rye clone' medium (III) with 0.54p m tryptophane in place of yeast extracts, and containing magnesium chloride and sodium sulphate.

µg. sulphate-S/50ml.

Passage number		Increase of main axis (mm.)	Lateral Number	Lateral length (mm.)	No. harvested
0	1	49.2 ±1.4	17.6 ±1.4	278±29	10
	2	47.2 ±2.7	15.3 ±0.9	274±22	10
	3	49.6 ±2.4	20.2 ±1.2	333±15	10
78	1	36.9 ±1.9	13.0 ±0.5	193±20	10
	2	37.0 ±2.9	13.7 ±0.7	221±52	9
156	1	40.5 ±2.0	14.6 ±1.2	202±18	10
	2	42.5 ±4.9	15.4 ±1.2	253±71	9
234	1	41.8 ±2.3	15.8 ±1.2	194±16	10
	2	41.2 ±2.0	14.1 ±0.4	196±18	8
468	1	34.5 ±2.4	15.2 ±1.6	208±30	9
	2	37.4 ±1.8	12.9 ±0.8	173±20	8
702	1	38.8 ±3.8	15.1 ±1.4	184±19	10
	2	41.2 ±1.9	11.8 ±0.7	124±19	9
Rye clone (III) with magnesium sulphate					
234	1	42.3 ±2.2	16.7 ±0.8	247±21	10
	2	44.5 ±1.8	16.2 ±0.9	194±13	10

		A Increase of fresh weight (mg.)	B Increase of dry weight (mg.)	B as percent of A	
	3	24.4 ±2.1			10
	4	16.8 ±1.2	1.1	7	10

Appendix Table 5 - 3

Growth of rye roots in 'rye clone' medium (III) containing 0.54ppm tryptophane in place of yeast extract with potassium nitrate, calcium nitrate and magnesium chloride replaced by their respective chlorides.

Medium as above with following additions and modifications.

First Passage.

Increase of main axis (mm.)	Lateral number	Lateral length	No. harvested
7mg. Sulphate as sodium sulphate/L. plus sodium nitrate (mg.nitrate/l.)			
33 31.1 ±1.8	14.1 ±0.6	236 ±33	8
66 33.4 ± 2.2	15.8 ±1.0	250 ±22	9
111 32.1 ± 2.4	14.1 ±2.1	262 ±48	9
166 29.7 ± 2.1	12.8 ±1.7	219 ±51	10
222 35.4 ± 1.7	17.9 ±0.9	331 ±22	7
333 36.2 ± 1.2	16.4 ±1.5	283 ±31	9
21mg. sulphate/L.			
33 32.9 ±1.4	16.2 ±1.3	276 ±22	9
66 31.3 ±1.2	13.3 ±1.3	217 ±22	10
111 27.6 ±1.5	11.5 ±1.6	195 ±28	8
166 35.2 ±1.6	14.1 ±1.1	258 ±28	9
222 35.4 ±0.6	17.9 ±1.9	279 ±35	8
333 34.5 ±1.4	15.9 ±0.7	266 ±23	10
14mg. sulphate/L			
111 32.0 ±1.4	14.9 1.0	283 ±21	9
magnesium chloride replaced with magnesium sulphate			
111 47.8 ±1.7	18.8 ±1.0	404 33	10

Appendix Table 5 - 3

Growth of rye roots in 'rye clone' medium (III) containing 0.54ppm trytophane in place of yeast extract with potassium nitrate, calcium nitrate and magnesium chloride replaced by their respective chlorides.

Medium as above with following additions and modifications.

Concentration of nitrate (mg./L) as sodium nitrate	Second Passage. B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
Concentration of sulphate (mg./L.)				
7 (as sodium sulphate)				
33	43.8 ±6.5	3.8	8.7	8
66	53.1 ±4.7	4.5	8.5	9
111	59.1 ±4.4	4.7	8.0	9
166	66.4 ±4.9	5.1	7.7	9
222	59.1 ±4.3	4.6	7.8	8
333	61.1 ±5.3	4.1	6.7	9
21 (as sodium sulphate)				
33	55.0 ±4.1	4.1	7.5	10
66	65.7 ±4.0	4.7	7.2	10
111	72.7 ±6.7	5.2	7.2	10
166	53.4 ±4.2	4.2	7.9	10
222	60.8 ±2.7	4.7	7.7	10
333	45.9 ±4.0	2.6	5.7	10
14 (as sodium sulphate)				
111	65.3 ±4.3	5.1	7.8	10
14 (as magnesium sulphate) magnesium chloride replaced with sulphate.				
111	28.8 ±3.0	2.3	8.0	10

Appendix Table 5 - 4

The Reduction of glucose concentration.

(Tryptophane) Medium (VI) with untreated glucose	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
Sugar concentration (per cent)					
2	1	16.0 ±1.2	1.4	9	9
	2	10.0 ±1.0	0.7	7	8
	3	26.2 ±2.1	1.7	6	7
	4	26.5 ±3.6	1.8	7	9
1.5	1	12.5 ±1.8	0.9	8	9
	2	4.5 ±1.2	0.2	7	9
1.0	1	9.1 ±1.1	0.6	8	10
	2	1.4 ±0.2	0.7	14	10
0.5	1	7.1 ±0.8	0.5	8	10
	2	2.3 ±0.5	0.2	9	9

The removal of sulphate-impurity Ion glucose.

(Tryptophane) Medium (IV) with ion exchanged glucose minus sulphate					
Flasks	1	11.7 ±1.1	1.0	9	9
chromed;	2	3.7 ±1.0	0.2	5	9
flasks cleaned	3	6.2 ±1.1	0.3	5	7
sodium hydroxide	4	6.3 ±1.9	0.5	7	6
flasks chromed	1	9.2 ±0.9	0.9	10	10
rinsed HCl;	2	2.6 ±0.3	0.2	8	9
flasks cleaned	3	4.5 ±0.7	0.3	7	9
sodium hydroxide;	4	3.6 ±0.5	0.2	6	9

Appendix Table 5 - 4

The Reduction of glucose concentration

(Tryptophane) Medium (IV) with ion exchanged glucose.	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
plus sulphate 234µg.S/50ml.	1	20.0 ±4.3	1.6	8	10
flasks chromed	2	10.7 ±1.1	0.7	7	9
	3	35.0 ±4.4	2.4	7	8
	4	33.0 ±4.9	2.5	8	10

Appendix Table 5 - 5

The growth of rye roots of several pH values.
'Tryptophane' medium (IV)
containing untreated glucose.

Initial pH value	Final pH value	Passage number	B Freshweight increase (mg.)	A Dry weight increase (mg.)	100A/B	No. harvested
4.0	4.7	1	13.4 ±1.6	1.1	8	8
	4.0	2	5.4 ±1.0	0.4	7	9
4.5	4.7	1	10.3 ±1.7	0.9	9	8
	4.4	2	8.4 ±2.0	0.6	7	7
5.0	5.1	1	17.3 ±2.6	1.4	8	9
	5.2	2	15.1 ±1.6	1.2	8	9
5.5	5.5	1	16.7 ±1.6	1.4	8	10
	5.6	2	19.7 ±3.3	1.5	8	10
6.0	6.1	1	22.4 ±4.3	1.8	8	9
	6.0	2	24.3 ±2.6	1.7	7	10
6.5	6.5	1	26.5 ±4.4	2.4	9	9
	6.4	2	32.7 ±4.1	2.5	8	10
7.0	6.6	1	12.8 ±2.0	1.2	9	8
	6.3	2	6.2 ±1.4	0.5	8	6
with ammonium nitrate replacing sodium nitrate.						
4.5	4.3	1	10.2 ±1.1	0.8	8	10
	4.2	2	7.0 ±1.2	0.5	7	10
6.0	4.3	1	19.0 ±1.5	1.5	8	10
	4.1	2	22.0 ±1.9	1.5	7	9

Appendix Table 5 - 5

The growth of rye roots of several pH values.

'Tryptophane' medium (IV)
containing untreated glucose.

Initial pH value	Final pH value	Passage number	B Fresh weight increase (mg.)	A Dry weight increase (mg.)	100A/B	No. harvested
with times five potassium phosphate concentration. ($100mg.\ KH_2PO_4/l.$)						
4.5	5.0	1	11.4 ±2.6	1.1	10	7
	4.5	2	9.3 ±2.4	0.6	6	8
6.0	6.0	1	27.0 ±3.1	2.3	9	10
	5.9	2	22.5 ±1.3	1.6	7	9

Appendix Table 5 - 6

The growth requirement of rye roots for thiamine
'Tryptophane' medium (IV)
with street's vitamins minus thiamine

Concentration of thiamine (µg./l)	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A / B	No. harvested
0	1	11.8 ±3.4	1.0	9	6
	2	0.7 ±0.4	0.2	3	7
5	1	18.0 ±2.7	1.8	10	9
10	1	13.0 ±2.3	1.3	10	9
	2	4.8 ±2.0	0.6	12	8
20	1	13.6 ±3.1	1.4	10	9
30	1	15.1 ±3.1	1.5	10	8

Appendix Table 5 - 8

The growth of tomato roots with several sulphate concentrations.

'Low N' medium (VI) minus sulphate with ion-exchanged sucrose

Sulphate-S µg./25ml.	Fresh weight gain (mg.)	Dry weight gain (mg.)	100A/B	No. harvested
0	6.6 ±0.8	0.72	10.9	8
0.33	7.1 ±1.3	0.64	9.0	8
0.66	6.8 ±0.6	0.73	10.7	8
1.0	9.8 ±0.5	0.88	9.0	9
1.66	12.5 ±0.7	1.16	9.3	8
3.33	14.0 ±0.7	1.14	8.1	9
6.66	17.9 ±1.3	1.40	7.8	7
10.33	15.0 ±1.3	1.21	8.1	7
26.6	17.4 ±2.8	1.45	8.3	7
53.3	15.5 ±2.1	1.25	8.1	7

Appendix Table 5 - 9

The growth of tomato roots with cysteine, methionine or sulphate

'Low N' medium (VI) minus sulphate

containing ion exchanged sucrose

µg.S/25ml.	Average fresh weight gain (mg.)	Average dry weight gain (mg.)	100A/B	No. harvested
with cysteine				
2	15.1 ±0.6	1.43	9.5	10
4	12.6 ±1.1	1.30	10.3	10
8	14.5 ±0.8	1.43	9.9	10
16	19.4 ±1.0	1.85	9.6	9
32	20.4 ±1.1	1.78	8.7	10
with methionine				
2	15.3 ±0.8	1.45	9.5	9
4	12.7 ±0.9	1.31	10.3	10
8	7.1 ±0.5	0.76	10.7	10
16	4.7± 0.5	0.58	12.3	10
32	3.1 ± 0.3	0.40	12.9	10
with sulphate				
0	13.1 ±0.8	1.30	9.9	10
8	14.5 ±0.6	1.51	10.4	10

't' for treatments 0 and 8µg. sulphate - S/25ml. is 1.43 for 18°F. N.S.

Appendix Table 5 - 10

The growth of tomato roots with either cysteic acid, taurine or sulphate

'Low N' medium (VI) minus sulphate containing ion exchanged sucrose

µg. S/25ml.	B Average fresh weight gain (mg.)	A Average dry weight gain (mg.)	100A/B	No. harvested
with cysteic acid				
2	12.7 ±0.45	1.13	8.9	9
4	10.0 ± 0.69	1.00	10.0	9
8	11.3 ± 0.94	1.10	9.8	9
16	14.3 ± 1.10	1.21	8.5	8
32	9.5 ± 0.36	0.88	9.3	9
with taurine				
2	10.1 ± 1.28	0.96	9.6	9
4	6.6 ± 0.58	0.75	11.4	9
8	5.9 ± 0.55	0.65	11.0	10
16	5.2 ± 0.75	0.60	11.5	10
32	4.1 ± 0.30	0.51	12.4	10
with sulphate				
0	7.1 ± 0.49	0.81	11.4	8
2.4	15.7 + 0.55	1.41	9.0	9
9.7	16.8 ± 1.06	1.49	8.9	8

Appendix Table 5 - 11

The growth of tomato roots with several sulphur sources.

'Low N' medium (VI)
containing 1.5% ion exchanged sucrose minus sodium sulphate

µg.S/25ml.	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A / B	No. harvested
0	15.5 ±1.6	1.61	10.4	8
Cystine				
2	18.5 ±1.9	1.75	9.5	8
4	11.9 ±0.9	1.28	10.8	8
8	12.9 ±0.6	1.38	11.5	9
16	15.5 ±1.8	1.56	10.0	7
32	22.6 ±1.1	2.13	9.4	6
Elemental sulphur				
2	10.3 ±1.8	1.14	11.4	8
4	10.3 ±1.8	1.12	11.0	9
8	9.6 ±0.7	1.03	10.5	8
16	12.2 ±1.1	1.25	10.2	9
32	12.8 ±0.4	1.35	9.7	8
Sulphate				
8	23.1 ±2.3	2.08	9.0	7
32	22.0 ±1.4	2.11	9.6	6
Containing untreated sucrose 1.5% plus sulphate				
117	20.9 ±1.4	1.97	9.4	
Plus sulphate and 0.2ml Acetone /25ml. medium				
117	22.1 ±2.7	1.95	8.8	

Appendix Table 5 - 12

(High magnesium) medium (VIII) - containing ion exchanged sucrose

Sulphate -S (µg./25ml.)	Passage Number	Fresh weight gain (mg.) B	Dry weight gain (mg.) A	100A/B	No. harvested
0	1	5.0±0.4	0.58	11.6	10
	2	2.9±0.4	0.35	12.1	10
0.3	1	7.9±0.5	0.83	10.5	9
	2	5.6±0.6	0.61	11.0	9
0.6	1	10.5±0.4	1.16	11.1	9
	2	8.5±0.5	0.89	10.5	9
1.0	1	16,1±0.6	1.43	8.9	9
	2	11.1±0.7	1.06	9.6	9
1.3	1	15.8±0.5	1.42	9.0	9
	2	12.7±0.6	1.18	9.3	9
1.6	1	16.8±0.6	1.47	8.7	9
	2	16.2±0.4	1.44	8.9	9
2.0	1	19.7±0.5	1.63	8.3	9
	2	17.6±0.5	1.44	8.2	8
3.0	1	22.2±0.6	1.84	8.3	10
	2	24.1±1.5	1.91	7.9	8
4.0	1	23.9±1.4	1.98	8.3	9
	2	27.5±0.9	2.23	8.1	9
5.0	1	26.0±0.8	2.32	8.9	7
	2	29.5±1.7	2.33	7.9	7
6.0	1	26.2±1.37	2.35	9.0	9
	2	27.8±1.0	2.32	8.3	9
7.0	1	28.1±1.0	2.38	8.5	9
	2	27.2±2.2	2.28	8.4	8

Appendix Table 5 - 12

(High magnesium) medium (VIII) - containing ion exchanged sucrose

Sulphate -S (µg./25ml.)	Passage Number	Fresh weight gain (mg.) B	Dry weight gain (mg.) A	100A / A	No. harvested
8.0	1	33.7±2.3	2.78	8.3	8
	2	25.2±1.6	2.24	8.9	7
16.0	1	27.0±1.9	2.35	8.7	8
	2	29.5±0.8	2.41	8.2	9
containing untreated sucrose					
117	1	28.6±2.6	2.49	8.7	7
	2	29.4±1.6	2.49	8.5	8

Appendix Table 5 - 13

'High magnesium' medium (VIII)-containing ion exchange sucrose

plus homocystine (µg. S/25ml.)	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
2	1	5.6±0.5	0.57	10.2	9
	2	2.7±0.4	0.36	13.3	5
4	1	6.2±0.5	0.62	10.0	9
	2	4.3±0.9	0.54	12.6	3
8	1	9.4±0.7	0.89	9.5	9
	2	4.6±0.3	0.51	11.1	10
16	1	11.7±0.7	1.14	9.7	10
	2	7.7±0.9	0.75	9.7	10
32	1	10.5±0.6	1.00	9.5	10
	2	3.9±0.7	0.49	12.6	9
plus Cystine (µg.S/25ml.)					
2	1	6.3±0.6	0.64	10.2	10
	2	4.1±0.5	0.46	11.2	10
4	1	7.1±0.3	0.73	10.3	10
	2	4.0±0.2	0.49	12.3	10
8	1	15.0±0.3	1.33	8.9	10
	2	13.2±0.6	1.24	9.4	10
16	1	12.7±0.4	1.18	9.3	10
	2	10.9±0.6	1.08	9.9	10
32	1	15.5±0.6	1.36	8.8	10
	2	17.7±1.0	1.59	9.0	10

Appendix Table 5 - 13

High magnesium medium (VIII) - containing ion exchanged sucrose

(µg.S/25ml.)	Passage Number	Fresh weight gain (mg.)	Dry weight gain (mg.)	100A/B	No. harvested
plus Methionine (µg.S/25ml.)					
1	1	20.7 ±0.6	1.63	7.9	9
	2	21.4 ±0.6	1.66	7.8	9
2	1	19.4 ±1.0	1.63	8.4	9
	2	18.2 ±1.1	1.57	8.6	9
3	1	20.6 ±0.8	1.63	7.9	9
	2	26.7 ±1.3	2.30	8.6	10
4	1	20.8 ±1.1	1.78	8.6	10
	2	27.1 ±1.5	2.27	8.4	10
6	1	19.3 ±1.3	1.68	8.7	9
	2	20.7 ±1.5	1.84	8.9	10
8	1	12.0 ±1.1	1.14	9.5	8
	2	15.0 ±1.9	1.51	10.1	8
16	1	7.8 ±0.6	0.85	10.9	9
	2	8.3 ±1.1	0.83	10.0	9
plus sulphate (µg.S/25ml.)					
0	1	6.0 ±0.7	0.56	9.3	8
	2	2.0 ±0.4	0.23	11.5	9
8	1	28.8 ±1.2	2.11	7.3	10
	2	30.2 ±1.9	2.58	8.5	9
16	1	29.2 ±1.6	2.14	7.3	10
	2	36.4 ±2.9	2.97	8.2	9
117	1	23.8 ±0.8	1.90	8.0	10
	2	31.6 ±1.9	2.65	8.4	7

Appendix Table 5 - 14

'High magnesium' medium (VIII) - containing ion exchanged sucrose

plus sulphate (µg.S/25ml.)	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
0	1	5.3 ±0.4	0.58	10.9	10
	2	1.8 ±0.3	0.20	11.1	9
8	1	31.6 ±1.5	2.40	7.6	10
	2	24.4 ±1.7	2.08	8.5	9
16	1	31.8 ±1.5	2.28	7.2	9
	2	26.6 ±1.3	2.21	8.3	10
plus cysteic acid					
1	1	4.6 ±0.6	0.48	10.4	9
	2	1.0 ±0.1	0.13	13.0	9
2	1	4.0 ±0.3	0.44	11.0	10
	2	1.4 ±0.1	0.14	10.0	10
3	1	4.1 ±0.4	0.47	11.5	10
	2	1.2 ±0.1	0.15	12.5	10
4	1	4.5 ±0.3	0.54	12.0	10
	2	1.7 ±0.2	0.22	12.9	10
6	1	4.2 ±0.4	0.46	11.0	8
	2	1.2 ±0.2	0.15	12.5	8
8	1	6.1 ±0.5	0.67	11.0	10
	2	3.0 ±0.5	0.24	8.0	10
10	1	5.5 ±0.3	0.60	10.9	10
	2	2.2 ±0.2	0.30	13.6	9
12	1	4.2 ±0.3	0.49	11.7	10
	2	2.0 ±0.2	0.22	11.0	10

Appendix Table 5 - 14

'High magnesium' medium (VIII) - containing ion exchanged sucrose

(µg.S/25ml.)	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
16	1	5.0 ±0.4	0.55	11.0	10
	2	4.2 ±0.7	0.44	10.5	10
20	1	5.6 ±0.7	0.59	10.5	10
	2	2.5 ±0.6	0.27	10.8	9
plus taurine					
1	1	5.5 ±0.5	0.54	9.8	10
	2	2.7 ±0.4	0.31	11.5	10
2	1	9.9 ±0.7	0.96	9.7	9
	2	5.7 ±0.5	0.57	10.0	9
4	1	7.8 ±0.5	0.77	9.9	10
	2	5.6 ±0.1	0.55	9.8	10
8	1	5.1 ±0.3	0.54	10.6	10
	2	3.0 ±0.4	0.32	10.7	10
16	1	6.4 ±0.7	0.64	10.0	10
	2	2.8 ±0.4	0.31	11.0	10
32	1	7.7 ±0.7	0.68	8.8	7
	2	3.9 ±0.9	0.32	8.2	7
with untreated sucrose plus sulphate					
117	1	34.8 ± 2.0	2.61	7.5	10
	2	29.7 ± 1.5	2.46	8.3	10

Appendix Table 5 - 15

The growth of tomato roots in 'high magnesium' medium (VIII) plus either methionine, cystine, filter sterilized double distilled water, or sodium sulphate.

'High magnesium' medium

µg.S/25ml.		B Average fresh weight increase (mg.)	A Average Dry weight increase (mg.)	100A/B	No. of roots harvested.
with untreated Sucrose 1.5% plus Sodium sulphate					
117	P1	27.8 ± 1.37	2.17	7.8	9
	P2	27.4 ± 1.95	2.38	8.7	8
with Ion-exchanged sucrose 1.5% plus sodium sulphate					
0	P1	3.9 ±0.45	0.44	11.3	10
	P2	1.8 ±0.25	0.22	12	10
1	P1	14.3 ±0.47	1.26	8.8	10
	P2	11.0 ±0.39	0.98	8.9	10
2	P1	18.1 ±0.82	1.43	7.9	10
	P2	16.4 ±0.48	1.33	8.1	8
8	P1	28.6 ±2.09	2.14	7.5	9
	P2	24.2 ±1.22	2.11	8.7	9
plus methionine					
0.5	P1	13.6 ±0.64	1.20	8.8	10
	P2	11.8 ±0.77	1.10	9.3	10
1.0	P1	17.2 ±0.65	1.54	8.9	9
	P2	13.0 ±1.04	1.25	9.6	8
1.5	P1	20.2 ±0.77	1.53	7.6	10
	P2	21.8 ±0.77	1.82	8.3	10
2.0	P1	26.4 ±1.47	2.06	7.8	10
	P2	25.4 ±0.94	2.10	8.3	10
2.5	P1	24.1 ±1.38	1.87	7.8	9
	P2	25.4 ±1.92	2.21	8.7	8

Appendix Table 5 - 15

The growth of tomato roots in 'high magnesium' medium (VIII) plus either methionine, cystine, filter sterilized double distilled water, or sodium sulphate.

'High magnesium' medium

µg.S/25ml.		B Average fresh weight increase (mg.)	A Average dry weight increase (mg.)	100A/B	No. of roots harvested.
3.0	P1	23.4 ±0.78	1.89	8.1	9
	P2	28.8 ±1.68	2.43	8.4	9
4.0	P1	18.5 ±1.30	1.57	8.5	9
	P2	23.4 ±1.72	2.00	8.6	8
5.0	P1	15.7 ±1.43	1.35	8.6	10
	P2	16.9 ±1.92	1.45	8.6	9
6.0	P1	13.4 ±0.98	1.25	9.3	10
	P2	11.6 ±1.39	1.07	9.2	10
with Ion-exchanged sucrose 1.5% plus cystine					
4	P1	7.0 ±0.76	0.7	10.0	10
	P2	4.2 ±0.68	0.43	10.3	10
8	P1	6.0 ±0.40	0.61	10.2	10
	P2	3.8 ±0.69	0.47	12.4	10
16	P1	10.0± 0.88	0.95	9.5	10
	P2	6.7 ±0.58	0.72	10.8	10
32	P1	15.3 ±1.47	1.42	9.3	9
	P2	14.3 ± 0.68	1.44	10.1	9
48	P1	18.3 ±0.47	1.60	8.7	10
	P2	18.0 ±0.67	1.66	9.2	10
64	P1	18.0 ±1.56	1.56	8.7	9
	P2	17.1 ±1.36	1.79	10.5	9
plus double distilled water ml. per treatment of twenty flasks					
2	P1	5.9 ±0.99	0.61	10.3	9
	P2	1.8 ±0.39	0.23	13	9

Appendix Table 5 - 15

The growth of tomato roots in 'high magnesium' medium (VIII) plus either methionine, cystine, filter sterilized double distilled water, or sodium sulphate.

'High magnesium' medium

µg.S/25ml.		B Average fresh weight increase (mg.)	A Average dry weight increase (mg.)	100A/B	No. of roots harvested.
4	P1	4.5 ±0.44	0.47	10.5	9
	P2	1.9 ±0.43	0.26	14	9
8	P1	3.4 ±0.26	0.35	10.3	10
	P2	1.8 ±0.42	0.23	13	10
16	P1	5.6 ±0.65	0.60	10.7	10
	P2	5.2 ±0.80	0.56	10.7	10
32	P1	4.3 ±0.44	0.45	10.5	10
	P2	2.1 ±0.29	0.26	12	10

Appendix Table 5 - 16

'High magnesium' medium - containing ion exchanged sucrose

µg.S/25ml.	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
0	1	7.2 ±0.5	0.74	10.3	10
	2	5.7 ±0.4	0.62	10.9	10
plus S-Methyl cysteine					
1	1	3.8 ±0.4	0.42	11.0	10
	2	1.3 ±0.2	0.16	12	10
2	1	3.6 ±0.3	0.37	10.3	10
	2	1.3 ±0.1	0.13	10	10
4	1	3.3 ±0.4	0.34	10.3	10
	2	2.1 ±0.2	0.23	11	9
8	1	2.2 ±0.2	0.27	12	10
	2	1.0 ±0.3	0.18	18	8
16	1	2.2 ±0.2	0.22	10	10
	2	0.7 ±0.1	0.17	24	9
32	1	1.0 ±0.1	0.11	11	10
	2	1.6 ±0.2	0.22	14	10
plus cysteamine					
1	1	6.6 ±0.5	0.67	10.1	10
	2	4.8 ±0.4	0.47	9.8	10
2	1	8.6 ±0.7	0.81	9.4	10
	2	4.3 ±0.6	0.43	10.0	9
4	1	4.4 ±0.3	0.48	10.9	10
	2	2.1 ±0.1	0.31	15	10
8	1	5.6 ±0.5	0.54	9.6	10
	2	2.7 ±0.2	0.35	13	10

Appendix Table 5 - 16

'High magnesium' medium - containing ion exchanged sucrose

µg.S/25ml.	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
plus cysteamine					
16	1	6.9 ±0.5	0.68	9.8	9
	2	3.7 ±0.6	0.47	13	10
32	1	9.4 ±1.0	0.91	9.7	10
	2	5.8 ±0.5	0.51	8.8	10
plus elemental sulphur					
4	1	6.3 ±0.8	0.69	11.0	9
	2	4.9 ±0.9	0.43	8.8	10
8	1	13.5 ±1.9	1.20	8.9	10
	2	8.6 ±1.3	0.93	11.2	9
16	1	21.3 ±0.6	1.69	7.9	9
	2	14.3 ±2.4	1.13	7.9	7
32	1	26.5 ±1.5	2.09	7.9	10
	2	23.3 ±1.0	2.02	8.7	9
64	1	33.8 ±2.0	2.62	7.8	9
	2	33.5 ±3.0	3.03	9.0	8
plus sulphate					
16	1	24.8 ±1.7	1.85	7.5	9
	2	32.8 ±2.7	2.89	8.7	8
-containing untreated sucrose plus sulphate					
117	1	31.7 ±2.7	2.23	7.0	10
	2	31.6 ±2.8	2.82	8.9	9

Appendix Table 5 - 17

'High magnesium' medium (VIII)
- containing ion exchanged sucrose

µg.S/25ml.	Passage Number	Fresh weight gain (mg.) B	Dry weight gain (mg.) A	100A/B	No. harvested
plus sulphate					
0	1	4.7 ±0.48	0.53	11	9
	2	1.96 ±0.3	0.09	10	9
1	1	13.5 ±0.2	1.18	8.7	10
	2	9.7 ±0.9	0.81	8.3	9
16	1	29.8 ±2.7	2.43	8.2	10
	2	28.1 ±1.4	2.41	8.6	10
plus (elemental S extracted with chloroform)					
	1	4.7 ±0.5	0.52	11	9
	2	3.0 ±0.4	0.35	12	9
plus distilled water extracted with chloroform					
16	1	5.8 ±0.3	0.63	11	10
	2	7.6 ±0.8	0.72	9.5	8
plus elemental S					
64	1	21.1 ±1.4	1.81	8.6	10
	2	16.1 ±0.6	1.49	9.3	9
plus glutathione					
1	1	5.5 ±1.2	0.55	10	10
	2	2.4 ±1.2	0.26	11	9

Appendix Table 5 - 17

'High magnesium' medium (VIII)
- containing ion exchanged sucrose

µg.S/25ml.	Passage Number	Fresh weight gain (mg.) B	Dry weight gain (mg.) A	100A/B	No. harvested
2	1	3.0 ±0.2	0.29	10	10
	2	2.9 ±1.9	0.28	10	10
4	1	4.9 ±0.3	0.44	9	10
	2	2.6 ±0.3	0.29	11	10
8	1	5.2 ±0.5	0.55	11	9
	2	2.2 ±0.4	0.25	11	9
16	1	5.3 ±0.4	0.50	9	10
	2	3.0 ±0.2	0.32	11	10
plus taurine					
1	1	6.3 ±0.5	0.69	11	9
	2	4.9 ±0.4	0.51	10	9
	1				
1.5	1	5.0 ±0.7	0.56	11	10
	2	1.7 ±0.3	0.22	13	10
2.0	1	4.1 ±0.5	0.46	11	10
	2	2.3 ±0.5	0.26	11	8
3	1	4.9 ±0.8	0.54	11	10
	2	1.3 ±0.3	0.15	12	10
4	1	3.6 ±0.4	0.38	11	10
	2	1.1 ±0.3	0.15	14	10

Appendix Table 5 - 17

'High magnesium' medium (VIII)
- containing ion exchanged sucrose

µg.S/25ml.	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
plus 1µg. sulphate -S / 25ml. and cysteic acid					
1	1	13.8 ±0.6	1.11	8.0	10
	2	11.1 ±1.0	0.92	8.3	7
2	1	13.9 ±0.5	1.25	9.0	10
	2	10.1 ±0.5	0.85	8.4	10
4	1	14.2 ±0.6	1.27	8.9	10
	2	10.5 ±0.8	0.88	8.4	9
6	1	15.4 ±0.6	1.36	8.7	10
	2	10.0 ±0.8	0.83	8.3	10
8	1	16.6 ±0.6	1.41	8.5	10
	2	13.2 ±0.9	1.01	7.7	10
-containing untreated sucrose plus sulphate					
117	1	27.7 ± 1.7	2.20	7.9	10
	2	30.9 ±2.0	2.55	8.2	10

Appendix Table 5 - 18

'High magnesium' medium (VIII)
- with ion exchanged sucrose

µg.S/25ml.	Passage Number	B Fresh weight gain (mg.)	A Dry weight gain (mg.)	100A/B	No. harvested
plus sulphate					
0	1	6.4 ±1.0	0.67	10	10
	2	3.7 ±0.9	0.33	9	9
16	1	25.6 ±0.9	2.05	8.0	9
	2	29.0 ±2.5	2.25	8.0	9
and plus 100ml. water extracted with chloroform					
16	1	26.5 ±1.0	2.06	7.8	10
	2	30.5 ±1.8	2.19	7.2	8
plus elemental sulphur					
64	1	25.2 ±1.2	2.14	8.5	10
	2	20.8 ±1.0	1.72	8.3	9
plus (elemental sulphur extracted with chloroform)					
0	1	7.1 ±0.5	0.74	10.4	10
	2	4.7 ±0.4	0.35	7.5	10
-with untreated sucrose plus sulphate					
117	1	26.3 ±0.8	2.05	7.8	10
	2	28.7 ±1.9	2.02	7.0	10